

Environmental protection or imperialism?

A year after the Rio summit, there are growing reasons to suspect that the generalization of environmental causes may place excessive burdens on very poor countries.

A YEAR after the UN conference on Environment and Development at Rio de Janeiro, some of the chickens are coming home to roost. As usual, there is some good news, some bad news and ample cause for alarm. The main business at Rio was the signature of the convention on global warming; enough of the signatories should have ratified the text for the treaty to be in force by the end of the year. That is the good news; the hope must be that in the years ahead, there will be enough sticks and carrots to win the compliance of governments that have not signed, and that understanding of the link between the accumulation of greenhouse gases and climate change will be, a decade or so from now, robust enough to foretell climate change and to compel avoiding action. That should be possible.

The bad news is that, as at Rio, the main goal is in danger of being overshadowed by less urgent, competing or even mistaken goals. The biodiversity treaty signed at Rio, described as the twin pillar (with the global warming treaty) on which the future will be built, may be a fine thing when there are criteria for deciding what resources should be spent to conserve what, but that is still some way off. Luckily, there is much that meanwhile can be done, by balancing the benefits of development against the risks of, say, soil erosion, to moderate and even halt the spoiling of pristine regions. That is also moderately good news.

Dangers

The cause for alarm is that the recognition at Rio that some environmental problems are literally global seems to have legitimized the belief that all environmental problems should be dealt with internationally. That is a mistake, potentially a dangerous one. For there are many important and still damaging environmental problems that are in no sense international. In much of the world, diseases caused by infectious organisms in the environment remain a persistent cause of death for example, but require investments in potable water supplies for their abatement. Newly developing countries seem to have a knack of creating unhygienic or otherwise damaging waste from industrial plants in a manner usually forbidden by law in prosperous places. Historically and by the contemporary comparison of rich and poor countries, it is plain that care for amenity is a function of prosperity.

To acknowledge that there are contrasts of this kind is not to suppose that they are tolerable. But it is a mistake to

pretend that poor countries can afford to live by the environmental standards to which rich countries aspire, worse still that they should be required to do so. The truth is that where people are relatively prosperous, they spend increasing proportions of the resources available on the avoidance of personal disaster (through insurance) and then on the avoidance of environmental risks. It makes no difference that much environmental protection is accomplished by regulation; if governments specify the purity of drinking water, the water utilities recover the costs from individuals, as either taxpayers or consumers. Environmental protection is thus often a public purchase on behalf of a whole community.

Resources

It is poor sense to expect that all communities can afford to live by the high environmental standards rich countries find congenial. To the extent that environmental protection costs resources, the expectation may even frustrate the economic development without which poor countries can never become rich. Yet the tenor of much post-Rio argument has been that in matters of the environment, there is one law for the rich and the same law for the poor. This is the spirit in which the World Bank is now required to stipulate that the development projects it supports must not damage the environment even when the consequences are likely to be confined to the recipient state. Europe's industrial revolution would not have begun if the same rules had been applied.

Rich countries in the nineteenth century were called imperialists because of their fondness for telling the poor how to run their affairs (and sometimes doing it for them). Ambitions to export the rich world's environmental standards to the poor may legitimately be understood in the same sense. Indeed, in the past few years, this new kind of imperialism has taken several sinister turns. The decision of the United States not to import tuna fish unless it could be shown that they had not been caught with nets that can drown dolphins was last year made illegal under the General Agreement on Tariffs and Trade (GATT). Now the future of the North American Free Trade Agreement awaits assurances that Mexico will not shelter behind relatively lax environmental standards to undercut the prices of US manufacturers (or even to tempt them south of the border). But Mexico, being relatively poor, may not be able to afford the environmental protection the United



States buys for itself.

That conflicts with the myth now being established that there is no essential conflict between environmental protection and economic or industrial development. (US Vice-President Al Gore helped the idea along in his speech at the United Nations last week.) In countries where environmental protection is already regarded as a public good, perhaps obtained by public purchase, that may be the case. By contributing directly to gross national product, the cost of environmental protection is on a par with, say, the production of motor-cars. But elsewhere, especially in the poor countries, the cost of environmental protection can be, and usually is, a drag. It will be a misfortune if the lingering enthusiasm for Rio gives this beguiling but misleading notion too wide a currency. In any case, the trouble between the United States and Mexico arises not because US citizens want Mexicans to enjoy their own amenities, but because they fear that Mexicans will steal their jobs. □

Graduates in limbo

Britain is not alone in its struggle to reorganize its research, but is again heading in the wrong direction.

THE question about the number of graduate students a country needs is like the more familiar conundrum about the length of a piece of string. It all depends, but chiefly on the purpose. Even so, the British government seems on the point of making a simple error in its planning for a new regime to cover the education of graduate students. Last month's White Paper (policy statement) said that courses leading to a research degree (a PhD or its equivalent) would ordinarily last for four years, the first of which would usually qualify a student for an MSc and would include course work of an appropriate kind. The idea was that a substantial proportion of students would find jobs outside academic life after their first year.

To the extent that the proposed first year would (among other things) help to identify people with aptitude and enthusiasm for research, the change is welcome. But from last Friday's debate on the White Paper in the House of Commons, it seems that Mr William Waldegrave, the minister responsible for the new Office of Science and Technology, has other things in mind. Students are to be taught management and industrial economics during this bridging period. And because the costs of graduate education will have to be quarried from the budgets of the research councils as they are now, larger numbers at the beginning of the four-year course will be offset by smaller numbers later on.

There are two dangers, on the first of which the present government has almost foundered in its relations with primary and secondary schools. Having laid down that there should be a national curriculum, the government then proceeded to specify what it should consist of and how the progress of students should be assessed. The plans for the

first year of graduate students' education sound very much the same. There is very little experience in Britain (or, for that matter, anywhere) of how best to combine preparation for research in, say, free electron lasers or genome sequencing with sufficient instruction in management and economics to make a student attractive to an industrial employer after a single year. The government seems confident that it can be done, but the universities have not yet tried to do it. Let us hope that this mismatch between expectation and reality does not degenerate into the mutual disrespect in which the schools and the education minister, Mr John Patten, hold each other.

The other difficulty is about numbers. Britain is already in a fix because it has too little of the skill (a blend of experience and daring) that people acquire during the course of a research degree. The annual output of 6,000 PhDs in science and technology is substantially reduced by the regular (although productive) loss to other professions, as well as by the drift overseas. Perhaps 4,000 people only are added to the domestic workforce each year. But then there is a further difficulty. The age-distribution of teachers at the older universities is skewed towards late middle-age, a monument to the great expansion of the 1960s. The newer universities, until recently called polytechnics, may at the same time be seeking to fill vacancies that arise with people of similar educational experience. The Association of University Teachers was right to argue the other day that there is likely to be a serious shortage of potential academics in the coming decade.

Can this then be the right time at which to be reducing the numbers of people trained in research? On the face of things, it seems the wrong time, especially because of the signs that shortages of people trained in research are also emerging in the fields from which pharmaceutical and biotechnology companies recruit. But that is only half of a sorry story. In all the discussions of the reasons for Britain's poor economic performance that preceded the White Paper, too little attention was paid to the capacity for enterprise of those who actually work as scientists in industry. And if — a common complaint — the economic climate is not conducive to innovation, may that not argue for a surplus of people able to play an active part therein? In short, there are reasons to fear that the government has misjudged this argument.

The difficulty is that the government should not be in the business of deciding how many graduates are needed in which fields, but should simply be concerned with the framework in which young people choose an education for themselves. Many able young people may be sufficiently put off by the government's new proposals to seek a graduate education elsewhere. That is another reason for suspecting that the government's targets may be too low. Of course, it is the research councils that will formally decide. The trouble with that is that the shortage of funds will give them an incentive to spend more on research and less on students. In everybody's interest, it would be better to hive off the student function to some body separate from the councils. □

Battle lines drawn for make-or-break vote on the future of the SSC

Washington. The Superconducting Super Collider will endure its darkest hour today Thursday (24 June) when the US House of Representatives votes on the Texas project's \$620 million budget allocation for the next financial year.

Formal presidential support for the collider, which finally arrived last week in a letter from President Bill Clinton to William Natcher (Democrat, Kentucky), chairman of the House appropriations committee, was greeted with relief by the 2,000 people already working at the SSC Laboratory near Dallas, but is unlikely to be decisive.

A defeat for SSC in the House of Representatives for the second year running could kill the project. But that outcome is not certain. Last year, support in the Senate and the connivance of a supportive House leadership unlocked the budget allocation.

On the other hand, if SSC survives this year, it is likely that the project will be completed. A total of \$1.5 billion has so far been spent, and its managers say that work is already 20 per cent complete.

This year support in the Senate, although likely, is by no means assured, and House leaders will have to consider the size of any majority against SSC before deciding how to proceed. George Brown (Democrat, California), the chairman of the House Science, Space and Technology committee, says: "The leadership in the House will decide after the vote how serious it would be if the Senate were to prevail again".

Meanwhile in Texas, four tunnel-boring machines are excavating one mile a week of the 54-mile tunnel that will house the underground collider, and work has just started on the two huge caverns for the detector equipment. Of the four booster stages that will accelerate particles into the main ring, the building has been completed for the initial linear accelerator and excavations have begun for the first two ring boosters.

A spokesman for the project, Russ Wylie, says the Clinton letter "gave everyone a real uplift", but adds that, in contrast with last year, when the House vote against the SSC had been a nasty surprise, this summer has been a time of "very deep concern" among researchers at Dallas. But Wylie says, "we think it's a winnable vote".

Top physicists have vigorously defended SSC in the face of increasingly virulent criticism from US politicians and commentators. "The SSC suffers from people not

having been down here", says Stan Wojcicki of Stanford University, California, and chairman of the Department of Energy's High Energy Physics Advisory Panel (HEPAP), who is spending a summer sabbatical at the SSC laboratory. "They view it as a paper project. But eight miles of tunnel have already been dug, and the laboratory is really active, holding three or four scientific meetings every week."

Wojcicki says that physicists who have attacked the project (who include his Stan-

no substantial foreign contribution and that "SSC supporters know it". Dale Bumpers (Democrat, Arkansas) will lead the attack on the project in the Senate, where the absence of strong support from former Texan senator Lloyd Bentsen (now Secretary of the Treasury) will diminish the project's chances. Last year, the Senate voted 66-33 for the project, while the House voted 232-181 to kill it.

The outlook for the project is further clouded by allegations of mismanagement of funds by Universities Research Association Inc, a consortium formed expressly to manage SSC. The House Energy and Commerce committee of John Dingell (Democrat, Michigan) will hold hearings on the matter next week. Friends of the collider say that the charges of mismanagement of funds concern only \$500,000 out of \$1.5 billion spent so far on the SSC, are in any case unproven and have been trumped up by SSC's enemies.

In his letter of support, Clinton said that "abandoning the SSC at this point would signal that the United States is compromising its position of leadership in basic science — a position unquestioned for generations."

But the political position of the president is likely to bear heavily on many Congressmen as they vote today. Some Democrats who opposed the project last year will rally to the new president, while Republicans may drift in the opposite direction.

But other Democrats, especially those in the 30-strong black caucus, may use the issue to vent their disapproval of recent moves by Clinton to appease conservatives with harsh public expenditure cuts that will hit the urban poor. If this improbable alliance of indignant liberals and fiscally stringent conservatives fails to kill the space station in a key vote expected on Wednesday this week, it will be doubly determined to bury the Superconducting Super Collider today.

Colin Macilwain

■ Hubert Curien, the former French minister of research, is expected to be elected this week (25 June) as the new president of the governing council of CERN, Europe's main laboratory for particle physics. Curien, a geophysicist, was president of the European Space Agency in the early 1980s, and would take up his new position on 1 January next year. One of the main tasks facing the new president is to negotiate political support for CERN's proposed Large Hadron Collider, the direct competitor to the SSC.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Construction continues apace under the Texan earth as Congress determines the SSC's fate.

ford colleague Ted Geballe), "are being short-sighted". He says that, if anything, other branches of physics are likely to lose money if SSC goes down.

But in the Congress last week, opponents of the project were moving in for the kill. The estimated total project costs, they say, have grown from \$4.4 billion in 1987 to \$9 billion last year, and Clinton's plan (in this year's budget) to "stretch out" the project by up to three years will raise costs by a further \$2 billion.

International collaboration has so far been restricted to contributions from Russia, India and China, which Wylie says are worth "a couple of hundred million dollars" in total. Japan is unwilling to get involved, at least until Congress gives its support this year. European support is similarly unsure; a decision on the competing Large Hadron Collider planned for CERN, at Geneva, is not due until the end of the year.

One House opponent says there will be

UK White Paper criticized for failure to switch from military to civil research

London. William Waldegrave, Britain's cabinet minister responsible for science, came in for criticism from both sides of the House of Commons last week for the failure of the government's recent White Paper (policy document) on science and technology to back any significant shift in funding from military to civilian research.

Prominent among the critics was Robert Jackson, who as parliamentary secretary in the Office of Public Service and Science (OPSS) — a post he has since lost in a cabinet reshuffle — was responsible for helping to oversee much of the drafting work on the White Paper. Speaking in a parliamentary debate, Jackson said that the government had missed the opportunity to address the potential which the ending of the Cold War offered for releasing the scientific and technological resources tied up in the defence industries. An industrial strategy was needed, he said, to ensure the rapid shift of these resources to competitive industries serving civilian markets.

Although the White Paper does contain a chapter on science and technology in the Ministry of Defence (MoD), this does little more than repeat the ministry's own views that the £2.6 billion (\$4 billion) it spends annually on R&D should primarily be devoted to the development of new weapons, and that it does not have responsibility for meeting broader national objectives.

The lack of any specific commitment to wealth creation, apart from emphasizing the need to encourage greater spin-off from MoD research projects, has given rise to the widespread suspicion that the chapter was not drafted by the Office of Science and Technology (OST) but by MoD officials.

It is also being quoted (for example by the Labour Party's shadow science minister, Marjorie Mowlam) as evidence that, despite being given responsibility for co-ordinating research spending across all government departments, OST may find it difficult to exert significant influence over how individual departments spend their money.

Those from the government's own party who had hoped for a stronger endorsement of moves to transfer funds from military to civilian research include Sir Giles Shaw, chairman of the Select Committee on Science and Technology. Shaw told the Commons that he was concerned that the White Paper did not provide the OST with any control over how the MoD uses its research funds — and that his committee was likely to look at this issue "very keenly" in the years ahead.

In reply to such criticisms, David Davis, who has taken over Jackson's responsibili-

ties in the OPSS, replied that he had been told it was "a good idea to be underestimated at the start of one's job".

Davis also said that the government was keeping a close eye on another concern raised in the debate, namely that, with the imminent splitting of the Science and Engineering Research Council, support for research in chemistry could suffer by falling between the responsibilities of the new Biotechnology and Biology Research Council, and those of the Engineering and Physical Sciences Research Council.

Both the Royal Institute of Chemistry and the Institution of Chemical Engineers have expressed concern that topics such as biological organic chemistry may find themselves neglected in this way — and that, unlike biologists and physicists, chemists will not have a research council they can describe as "home". Davis responded by promising to keep the issue under review, and pointing out that all the research councils had been told to ensure support for the

"underlying science" in their areas of responsibility.

David Dickson

■ The setting up of Britain's new Council for Science and Technology — the replacement for the Advisory Committee for Science and Technology (ACOST) — has been brought forward by two months to speed up the implementation of recommendations in the government's White Paper.

William Waldegrave said last week that next month's meeting of ACOST would be its last, and that the new CST, whose members will include industrial and academic scientists as well as representatives of government departments, will be established by the end of October.

According to Waldegrave, the accelerated schedule (CST was originally to be set up by next January) will allow the council to play an active role in the preparation of the government's first "forward look", setting out the five-to-ten-year prospects for the research programmes of individual government departments.

Duesberg's anti-AZT campaign continues

London. Peter Duesberg, the controversial US molecular biologist who claims that the main cause of AIDS is recreational drug use rather than HIV infection, has rejected as a "fabrication" the results of a recent study claiming to disprove his hypothesis.

A group of US researchers, led by Michael Ascher of the California Department of Health Services, have used data from the San Francisco Men's Health Study to show that men who were heavy drug users but showed no evidence of infection with HIV did not contract AIDS, in contrast to those in the study who were either light drug users, or did not use drugs at all, but were shown to be infected with HIV (*Nature* 362, 102; 1993).

The California researchers argue that the results directly challenge Duesberg's idea that AIDS is a clinical manifestation of long-term consumption of recreational drugs and of treatment with the antiviral agent zidovudine (AZT). However Duesberg himself, speaking at a meeting in London last weekend organized by a group known as the Steering Committee Against AZT Malpractice (SCAM), said that he refused to accept the group's conclusion, and continued to insist that his own interpretations are valid.

In particular, Duesberg criticized the labelling of one table used in the publication of the California group's results for not indicating the category of "no drug use", and that the paper was therefore invalid (apparently disregarding an explanation in the text that these subjects were represented

in the table's category of "light" drug users).

He also said that he refused to accept the way that the results of the study were presented in a graph, claiming that "the curve is a fabrication and the conclusions are flawed". Indeed, Duesberg claimed that the research group's findings could be interpreted to support the opposite conclusion to that suggested, namely that there was a 100 per cent correlation between AIDS and drug use.

In support of his own views, Duesberg told the meeting that AZT was as toxic to human T-cells (the target for HIV infection) as it was to the virus itself. But this, he said, is ignored by Wellcome, the company which produces AZT, as well as by researchers and doctors who are currently treating HIV-positive patients with AZT, and who follow earlier studies by Samuel Brodeur and colleagues which showed that human cells are 1,000 times less sensitive to AZT toxicity than HIV.

Michael Ascher, speaking by telephone from California, was "stumped" to hear that Duesberg was still trying to publicly misrepresent the *Nature* epidemiology study, particularly when Duesberg has acknowledged in a letter to *The Lancet* (12 June 1993) that the category of "light" drug users mentioned in the study includes the "none" drug users. Ascher said that Duesberg appeared unable to accept clear controlled epidemiological data and simply jumped from one favourite hypothesis to another to suit his argument.

Julie Clayton

Mood of space station partners lifts

Paris, Washington and Tokyo. Space station supporters were confident of winning the support of the US House of Representatives in a crucial vote due yesterday on President Clinton's revised design proposal for a version of the project which will cost \$10.5 billion over five years.

But after the president announced his own support for the project last week, it emerged that no detailed work has been done on the proposed compromise design

modular, 'tin can' option thought to be favoured by NASA administrator Dan Goldin has been ditched, much to the relief of the international partners. The partners are even more relieved that Clinton has given a space station design his full backing, and are now confident that the project can regain some of the momentum it has lost in the past few months.

"We couldn't have hoped for a better outcome," says Lanfranco Emiliani, head of the European Space Agency's ECU 2.4 billion input to the programme.

Clinton's endorsement of a space station combining elements of options A and B has allayed ESA's fears for the project's future. ESA is also pleased that Clinton deferred a decision on the orbit. The president's special advisory panel on the station redesign, chaired by Dr Charles M. Vest, president of the Massachusetts Institute of Technology, had recommended the higher orbit. But ESA was worried that launching its module into this orbit would require using the Russian Proton rocket, a competitor of Ariane.

Clinton's decision has restored some measure of confidence among the space lobby in Europe, where support for the station has been crumbling. "There is now the political will to continue with the space station," says Klaus Berge, director of the German space programme. Whether there is a financial way is another matter. Italy, for example, has already said that it cannot afford to meet the commitments it made in Granada last November (see *Nature* 360, 199; 1992).

Continued support for the station in Europe is likely to be forthcoming only if the present ad hoc collaboration is transformed into a truly international project. The stumbling block to a *ménage à trois* between Europe, Russia and the US is that all three are competitors — for example in the commercial satellite launch market (see below).

And while Europe and the United States make much of their efforts to save Russian space competencies in public, officials admit in private that both are jealously competing for access to Russian technology.

Russian prime minister Viktor Chernomyrdin is likely to discuss greater Russian participation in the space station when he meets US vice-president Al Gore next month. ESA objects to any broader Russian role now, but Jean-Marie Luton, director general of ESA, says that he is open to "a redefinition of the international space station" if this is "acceptable to all the present partners."

Relief at the demise of option C is shared by Japan. According to Junji Yoshihara, director of the office of space utilization of the Science and Technology Agency, the government agency funding Japan's space station programme, Japan would be happier with B but they can live with A as well.

Japan is already committed to spend half of the ¥310 billion (\$2.95 billion) it plans to spend on its contribution to the station. In addition, it has spent ¥4.6 billion so far on a new control centre for the station in Tsukuba science city. Construction of the Japanese module is due to start this year. Yoshihara says that the agency had hoped to increase the budget for the station by ¥20 billion next fiscal year (beginning 1 April) from this year's ¥45.8 billion. "But because of the redesign, the Ministry of Finance is asking 'if the United States can reduce its budget for the station why can't we?'".

But Japanese officials recognize that, despite Clinton's commitment, the final decision will still rest with Congress, and that the station could still be dropped — a prospect for which they have prepared no contingency plans. Last week, they were continuing to warn of the dire consequences of such an outcome for international scientific collaboration.

**Declan Butler, Colin Macilwain
and David Swinbanks**



Luton: open to "a redefinition of the international space station".

which, the White House says, will reconcile aspects of the two modular space stations — options A and B — suggested by a NASA redesign process, while costing slightly less than either one.

"I sometimes wonder if they've sort of fuzzed it up to try and keep me happy", says George Brown (Democrat, California), chairman of the House Science, Space and Technology committee and salesman-in-chief for the space station in Congress. Brown admits it will be hard to sell a compromise that remains so ill-defined. "It would be better if we had the information, but we won't get it for two or three months", he says. However, as they canvassed for support late last week, advocates of the station were confident of winning yesterday's vote.

The White House has also deferred judgement on which orbital inclination the space station should be launched into — the 51.6 degrees which would suit Russian launchers, or the 28.2 degrees for which the US space shuttle is designed. Brown has even floated the idea of a 'compromise' orbit between the two: the Russian orbit is constricted by its concern about flying over China, which might be readily eased.

What is clear is that Option C, the non-

Launch wars

Even as Europe, Russia and the United States discuss prospects for greater cooperation in the space station, all three are engaged in a bitter battle for a share of the commercial satellite launch market.

The United States and Europe are concerned that unless Russia's entry is regulated it could destabilize the market through what they see as 'dumping' — Russia can undercut by half the \$62-million price-tag of an Ariane launch. But the United States and Europe are divided over the rules of fair play: the United States accuses Europe of subsidizing Ariane, while ESA wants the United States to open up government contracts to foreign competition.

Independently of Europe, the United States this month agreed tariffs and quotas with Russia for the launch of US satellites using its Proton rocket (Lockheed has also agreed with the Krounitchev company to market Proton in the United States). Europe immediately accused the United States of manipulating events to destabilize Ariane as the market leader (50 per cent) while protecting its own Atlas, Titan and Delta launchers. But ESA has since made a similar agreement with the Russians.

D.B.

Japan's protected wetlands grow under NGO pressure

Tokyo. The Japanese government won qualified praise after hosting the world's leading forum for the preservation of wetlands and waterfowl, but on past performance the good intentions of Japan's environmentalists may be overwhelmed by the pressure for development.

The occasion was the fifth meeting of the Ramsar wetlands convention, the treaty drawn up in 1971 at the Iranian city of Ramsar, and now ratified by 77 governments. This year's meeting was at Kushiro, on Japan's northern island of Hokkaido, from 9 to 16 June.

In a move unfamiliar in this country, Japan's fledgling non-governmental organizations (NGOs), such as the Japanese branches of the World Wide Fund for Nature (WWF) and Friends of the Earth, banded together before the conference to push for better protection of Japan's own rapidly disappearing wetlands.

Tidal flats around Japan are important resting and feeding points for migratory birds and are also a vital resource for fisheries. It is reckoned that, during the long postwar economic boom, more than half of Japan's natural coastline on the four main islands disappeared under concrete walls, sea defences and land reclamation projects. The trend continues: the government's Environment Agency let slip during the Kushiro meeting that, between 1979 and 1985, Japan lost another 4,000 hectares (or more than 7 per cent) of its remaining tidal flats.

Before the Kushiro meeting, only four wetland sites in Japan, totalling 10,000 hectares, were listed for protection under Ramsar. No rich nation has fewer. Worldwide, more than 600 sites covering more than 38 million hectares have been designated by the 77 members of Ramsar. Britain, for example, has nominated 60 sites covering more than 270,000 hectares. And none of the Japanese sites is on the coast.

The NGO coalition at Kushiro pushed the Japanese government hard to list more sites, particularly tidal flats. It had in mind such sites as the Wajiro tidal flat in Hakata Bay, on the southern island of Kyushu, where the local government plans to build an artificial island in the middle of the bay, and Fujimae tidal flat near Nagoya, which may be used as a garbage dump. Although it failed to get any tidal flats listed, the Japanese government did add five more inland sites (the largest being Lake Biwa near Kyoto) totalling more than 70,000 hectares to the Ramsar list.

One of the aims of the conference was to draw up guidelines for the "wise use" of wetlands. A draft presented at the meeting called for mandatory environmental impact

assessments of proposals for development projects at Ramsar sites. But Japan, which has no legally binding system of environmental impact assessment, balked. Instead, it insisted that the guidelines should "endorse", but not "require", assessments.

Such impact assessments as there are in Japan are usually carried out by the organizations responsible for development projects, and almost invariably favour development. Ramsar delegates and NGOs are particularly concerned about plans by Japan's Construction Ministry and the Hokkaido Development Agency to build a huge flood control channel in the watershed of the

Bibi river, which flows into Lake Utonai in Hokkaido; one of Japan's Ramsar sites. They fear it could alter water levels. Under the gaze of the media at Kushiro, Japanese government officials promised that there will be a proper environmental impact assessment of this project.

Ramsar governments also agreed to double the convention's annual budget to US\$1.5 million for the next three years. The Japanese NGO contingent is particularly happy that Japan's Environment Agency has agreed to form a committee of experts, including NGOs, to advise on protection of wetlands.

It remains to be seen if the Japanese government will live up to its promises to protect its own Ramsar sites. The Environment Agency seems determined to do so, but the little agency, with little political power and a tiny budget, is often overruled by more powerful ministries with other interests.

David Swinbanks

New boss for Russian research foundation

Moscow. To much surprise, Andrei Gonchar, vice-president of the Russian Academy of Sciences (RAS), is not to be the chairman of the Russian Foundation of Basic Research, the Russian government's new instrument for making grants to researchers.

Although Gonchar was named last year as director-organizer of the foundation (RFBR), a decree now signed by President Boris Yeltsin says that the job will instead go to 46-year-old Vladimir Fortov, a research director at the RAS Institute of High Temperature Physics.

Although Gonchar has been considered the most likely chairman, his official appointment has been repeatedly postponed for the past six months. The explanation is that Gonchar has refused to give up being vice-president of the RAS. The charter of RFBR expressly prohibits its chairman from occupying any other leading position at the academy. The government and the Ministry of Science are also determined to keep the foundation and academy separate.

Andrei Gonchar has repeatedly said that he cannot resign as RAS vice-president because that is an elected position, and that if he had to choose between the academy and the foundation, he would remain at the academy. But, judging from the negative reaction of the academy leadership to the appointment of Fortov, Gonchar never seriously considered that one line in the charter would disqualify him as chairman of the foundation. Indeed, in December 1992, he insisted to the press that the RFBR Charter does not mention the possible conflict of interest, suggesting that Gonchar had not read this document, then published for all to read.

But Yeltsin's failure to appoint Gonchar does not reflect disapproval of his work as the leader of RFBR during its first year. First Deputy Minister of Science Andrei

Fonotov rates Gonchar's achievements highly. Chiefly, he made the RFBR the first really functioning research foundation in Russia.

The foundations's first grants were of unquestionable value to Russian science. One of the foundation's experts, Konstantin Kikoin of the Kurchatov Institute, says that Gonchar was able (at least in the field of physics) to gather a qualified team of experts, and did everything possible in order to ensure that grants were awarded to the very best. At the RAS Institute of Physics, the St Petersburg Physical Technical Institute, Nizhny Novgorod (formerly Gorkii), Kazan, and many other provincial scientific centres in Russia, Kikoin says that "science woke up."

But the foundation's activities have also been the cause of some criticism. Gonchar was at best tactless when he awarded grants to practically all members of the praesidium of the RAS, as well as to all the foundation's own appointed experts.

The main criticism is that the foundation mainly concerned itself with work performed within the framework of the RAS. Andrei Fonotov says that, according to his data, 85 per cent of the grants went to academy laboratories, where most of the foundation's experts also work. Perhaps a half of Russia's basic research has thus been outside the foundation's gambit.

Resentment of this emphasis on the academy's own scientists has been expressed by such non-academic institutes and associations as the St Petersburg Union of Scientists, the Russian Physical Society and the Russian Astronomical Society. The initiative to replace Gonchar was eventually taken by Boris Saltykov, the minister of science. Vladimir Fortov was chosen after lengthy discussions.

Vladimir Pokrovsky

Doctors' dilemma paralyzes French medicine

Paris. Legislation intended to outlaw conflicts of interest among French physicians in the prescribing of drugs for patients has spread confusion through the medical profession and raised the possibility that biomedical research will suffer. Under a new law passed by the previous Socialist government, physicians who accept any form of payment from drug companies can be fined FF500,000, jailed for two years and struck off for ten. To be safe, physicians have no doubt taken to buying their own ballpoint pens.

The purpose of the law is to discourage physicians from partisan prescribing, considered bad for patients and the health deficit. But medical organizations and the drug companies say the law will also paralyze science. Much of the criticism can be traced to the medical profession's unhappiness at being tainted with suspicion because of the actions of a few.

Controversy centres on an exemption for activities whose "real purpose is research or scientific evaluation". Physicians particularly resent a condition that the Conseil National d'Ordre des Médecins (CNOM) should approve every transaction, protesting that bureaucracy will stifle spontaneity. CNOM, which backs tighter control, has introduced standard contracts and other simplifying measures.

The exemption's real flaw, says Pierre Colombier, a physician at the federation of continued medical training associations UNAFORMEC, is that CNOM's approval will not guarantee immunity from prosecution. "It's not yet clear what's permitted, and what's not," says Jean-François Sforti, president of Sanofi-Winthrop. Drug companies, which would face stiff penalties as accomplices, say that this ambiguity has left them with no choice but to call a moratorium on scientific activities involving payment or perquisites.

It is too soon to say if the law will adversely affect science. Most of this year's activities were arranged before it came into force, and are unaffected. But the potential for damage is large. The pharmaceutical industry informally sponsors around FF20 billion's worth of scientific activity every year (about FF 100,000 per physician), although Colombier says that much of this goes on marketing exercises.

But the new law will certainly hurt scientific conferences, many of which are sponsored by drug companies, which sometimes pay physicians to attend. Although some are alibis for holidays, most are of high-quality and thus are essential to cross-fertilization between clinicians and researchers. Many conferences have already been cancelled (and hotel labour unions are complaining). The pharmaceutical industry and CNOM are asking that attendance at conferences

should be free from control.

Many post-market drug studies (so-called Phase IV trials) will also be hurt, although their scientific value is often dubious. They frequently lack placebo controls or neglect to blind investigators, and appear sometimes designed to acquaint physicians with particular products. There have been cases where physicians can trade a certain number of prescriptions for a case of champagne. Phase I, II and III clinical trials will not be affected, as these are already strictly regulated.

Drug promotion, which the new law all but bans, is a grey area. Physicians need to learn about the latest drugs, and how best to use them. "The possibilities of fraudulent agreements are even more tempting when he who buys the product is neither he who pays, nor he who uses it", reflects *Prescrire*, a magazine that refuses drug advertising on

its pages. CNOM and pharmaceutical unions want the law to be brought into line with a 1992 European directive on the matter, which allows for "reasonable hospitality" (to physicians by drug companies).

The new government has now promised a more flexible interpretation of the law, but major changes would require new legislation and would take time. The government will not wish to be perceived as soft on corruption. So the physicians' dilemma may be resolved less formally. Roger Luccioni, professor of cardiology at the La Temone hospital in Marseilles, has helped create a nonprofit clearing-house for funds from drug companies. Benign neglect of the provisions of the law, as with the recent restrictions on smoking in public places, is another remedy: although the smoking restrictions are not enforced, people appear to be smoking less.

Declan Butler

British group backs French scientist

London. A British ethics committee has given its support to Jean-Pierre Allain, one of the three specialists from the French National Blood Transfusion Centre (CTNS) currently appealing against their convictions by a Paris court for allowing haemophiliacs to be treated in the mid-1980s with blood infected with HIV.

The committee, chaired by the philosopher Baroness Warnock, has concluded that

awaiting the outcome of his appeal, expected on 13 July. He has long protested that he has been made a scapegoat for decisions (to delay the introduction of heat treatment) by his superiors both inside and outside the CTNS (see *Nature* **363**, 491; 1993).

Warnock's committee was set up by the East Anglia Regional Health Authority to decide whether Allain was fit to continue in his position as director of the regional transfusion centre. In a report completed last month — but whose publication was held back until the appeal hearing ended in Paris last week — the committee says that, judging his actions "in the light of the scientific climate of the time", there is "no reason" to regard him as unfit to hold his Cambridge position.

Christian Voulard/Gamma/ISG

IMAGE
UNAVAILABLE
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REASONS

Allain (right) with co-defendants in French AIDS trial.

Allain acted in a way "consistent with medical professional ethics" in warning his superiors of the dangers of allowing haemophiliacs to be given potentially contaminated blood, and urging the rapid adoption of foreign heat-treatment technology.

Allain, who is now a professor of transfusion medicine at the University of Cambridge, has been suspended at his own suggestion as director of the East Anglian regional blood transfusion service while

Allain said last week that he felt the committee's conclusions "gives me back my integrity and honour". But he is bitter about the lack of support from his French colleagues, claiming that many of them are "scared" to come to his defence in the light of widespread criticism of their own actions in not warning haemophiliacs of the dangers of untreated blood.

The regional health authority is awaiting the outcome of Allain's appeal before deciding whether he should continue to be suspended from his position with the blood transfusion service. Meanwhile Allain is continuing with his research at the university.

David Dickson

UK government's support 'essential' for exploitation of superconductivity

London. Less than a month after the British government outlined a new strategy for linking science to wealth creation, its strategy is being tested by demands for support for the development of new products based on recent advances in superconductivity.

Leading researchers, in both universities and industry, claim that extra government support is essential to bridge the persisting gap between the technological and commercial potential of superconductivity. Without it, they argue, British companies will

DTI. One team looked at developments in the power engineering applications of both low- and high-temperature superconductivity, the other at their applications to electronics systems.

Both teams were impressed by what they had been shown at government, university and industrial research laboratories. They were also convinced that, even at a lower level of funding, British achievements remain comparable with those in the United States: "the green shoots are there," says Gordon Donaldson, professor of physics at the University of Strathclyde and the leader of the electronics team.

But both teams also argue in their separate reports, published by the institute last week, that while the US government continues to provide full support for many industrial research projects — currently spending about \$400 million a year — the recent rearrangement of funding for technology casts doubt on the prospects of a similar type of support for superconductivity in Britain.

The main need at present, according to superconductivity researchers, is for backing for a number of "demonstrator" projects to prove the commercial viability of products incorporating superconducting technology, for example in passive microwave devices that can be used for satellite communications.

But each of the more obvious routes forward outlined in the government's white paper (policy statement) last month presents difficulties. For example, research groups in at least two large companies — ICI and GEC — have already achieved significant results in areas such as the production of superconducting thin films and logic devices. Yet in neither case is the parent company so far prepared to make a unilateral commitment sufficient to turn these developments into marketable products.

The strategy popular in the United States of using small start-up companies as the cutting edge of product innovation is widely seen as more difficult in Britain, both because of the greater difficulty in raising venture capital and the relative lack of the government contracts on which many start-up companies rely.

One significant source of funding is the Science and Engineering Research Council (SERC), which has itself been funding a major superconductivity research programme. But moves to shift more SERC funds (or, more particularly, those of its planned successor, the Engineering and

Physical Sciences Research Council) into product development are likely to meet resistance from other scientists, arguing that it would inevitably lead to reduced funding for basic research.

And although the government is encouraging companies to collaborate at the European level through programmes organized by the Commission of the European Communities in Brussels, researchers in the field argue that the 12–18 months required for such an initiative to get off the ground is too long to meet the immediate needs of British industry.

The solution being put to the government is that it should back a three-year joint initiative, involving the DTI, the SERC, the Defence Research Agency and industrial participants, and costing an estimated £2 million a year over this period. The programme would aim at setting up inter-company teams with the goal of targeting specific market sectors.

Donaldson, whose own work pioneering the medical applications of superconducting detector coils is already being taken up by US companies, argues that such a programme is essential if a British superconductivity industry is to take off. "At present, the industry is at a crisis point; we must act now," he says.

So far, there has been no positive response from the DTI, although officials say that they are keeping the situation "under observation" — and point out that potential avenues for continued funding still exist through, for example, the LINK scheme, by which it sponsors joint projects between industrial and academic researchers.

The SERC has reaffirmed its own support for superconductivity research. The topic figured prominently during a two-day meeting of its materials commissions last week, which decided to maintain funding for such research at its current level of about £3.4 million a year.

But in a situation where everyone seems to be waiting to see who blinks first, fears are growing that superconductivity in general, and high-temperature superconductivity in particular — both fields to which Britain has made fundamental scientific and technological contributions — could join the list of technologies that end up being exploited elsewhere.

"Superconductivity is like a child which is growing into adolescence," says David Caplin, professor of physics at Imperial College, London. "It has grown through an immensely successful childhood stage, and has already shown its future potential; but it still requires parental support if that potential is to be realized."

David Dickson

IMAGE
UNAVAILABLE
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REASONS

A superconducting filter (right) could replace the bulkier conventional filter (left) in picking up radio signals from satellites.

inevitably fall behind their competitors in the United States and Japan, many of whom were able to benefit from substantial government backing.

But the Department of Trade and Industry (DTI) has decided to axe the Advanced Technology Programme (ATP), under which it has spent £8 million over five years on the applications of superconductivity. That follows from the department's decision to shift the focus of its support for innovation towards the mechanisms of technology transfer, and thus away from direct backing for new developments.

Calls for government action have been stimulated by visits to the United States of two teams of British physicists, organized by the Institute of Physics and funded by

Pragmatism keeps Berlin centre alive

Berlin. A plan to keep in being a group of research institutes at Adlashof, a suburb of southeast Berlin, is likely to be agreed next month. This development should make good the most conspicuous flaw so far to come to light in the otherwise successful programme for reshaping the pattern of research in the East masterminded by Germany's science council (Wissenschaftsrat) during 1991.

Before reunification, the 50-hectare Adlashof site was occupied by nine chemistry and physics institutes of the former Academy of Sciences and a large centre for making western-type instruments. The original

had enough to handle with their own restructuring and staff reductions (see *Nature* **362**, 775; 1993), while the idea of moving a whole institute hundreds of kilometres was clearly impractical.

But financial quarrels proved decisive. If the centre were a university institute, the *Land* (state) of Berlin would be responsible for the whole cost, but as an independent research institute, the federal government would have to share the expense. Nobody in Berlin was convinced that an expensive chemical research centre in a city without a chemical industry could be a priority. "The *Land* refused to take it on", says Jochen Stoer of Berlin's ministry of science and art. So the staff at the chemistry centre are being paid from a special fund (called WIP) intended to support individual scientists moving from institutes to universities, but that fund will end in December.

Various contingency plans have been cooked up and rejected. Then, last February, the Wissenschaftsrat was asked to try again. It asked for more staff cuts (down to 220) and suggested that the

centre should become a 'Blue List' institute, meaning that the *Land* and federal governments would split the costs equally. But the Wissenschaftsrat said it would not make that proposal formally until the two governments had agreed to share the costs.

This was a wise precaution. Had the suggestion to fund a Blue List institute been made at the outset, it would certainly have been accepted. But now the federal government believes that too many such institutes have already been founded in the new *Länder* (see *Nature* **363**, 482; 1993). Finance minister Theo Waigel has enunciated the omnibus principle: no new Blue List institute unless an old one is closed.

Months of carrot-and-stick negotiation may nevertheless now bear fruit. The centre has won support from the previously sceptical Berlin science minister, Manfred Erhardt, who seems this month to have reached an understanding with federal research minister Paul Krüger (himself an east German). And the federal research ministry is prepared to pay half the cost of the centre for ten years provided that it is not formally established as an institute of any kind.

Berlin will match federal support alone, without calling on the other five eastern *Länder* to which Blue List status would have entitled it. But Berlin will administer the centre just as it does its eight Blue List institutes. The federal and Berlin governments are believed ready to accept this pragmatic solution after it is formally proposed by the Wissenschaftsrat on 9 July.

Researchers at the Chemistry Centre

welcome the new concept of an institute in fact if not in name. "Ten years is a very long time for us to prove our worth", says department head Manfred Meisel. With nearly 600 publications and 27 patent applications last year, they have reason to be confident.

Alison Abbott

NIH appointment due

Washington. The Clinton administration is believed to be considering two very different candidates to direct the National Institutes of Health (NIH), the United States' main biomedical research institution.

Dr Judith Rodin, a psychologist and provost of Yale University, and Dr Harold Varmus, a Nobel Prize winning cancer researcher from the University of California at San Francisco, are being considered to replace Bernadine Healy as director of the NIH. Healy, who was appointed to the post by former president George Bush, is to vacate the position by 1 July.

While both Rodin and Varmus come highly recommended by a search committee of scientists, they would each be likely to emphasize different roles for the government-funded NIH, whose budget this year will exceed \$10 billion. Rodin, 48, is an experienced administrator and a behavioural scientist known for her research on aging and eating disorders. She would be expected to bring a social slant to the agency's approach towards research, emphasizing, for example, disease prevention and women's health issues.

For his part, Varmus has heavy ties to the biomedical research community. Although trained as a doctor, Varmus, 53, has worked primarily in molecular biology, winning the 1989 Nobel Prize for his research on the genes that cause cancer. He has no administrative background, but has taken an active interest in science policy and has testified several times to Congress on biomedical research. Insiders say that Varmus's nomination has been recommended to President Clinton by Health and Human Services Secretary Donna Shalala, who oversees the NIH.

The selection process has been less rocky so far than the last time an NIH director was chosen. In 1991, Healy, a cardiologist, was chosen after several candidates turned down the job because, under the Bush administration, they would have had to publicly oppose abortion and fetal research.

"We're not seeing any ideological litmus testing going on in this process," said Lynn Morrison, director of public policy for the American Federation for Clinical Research, a New Jersey-based lobby group. "The administration is trying to find the right person to run the NIH, not the right person for its particular political stance." **Susan Greene**



Relieved: the Chemistry Centre in east Berlin.

workforce of 5,500 workers has already fallen to 3,800 and will fall further, by a third, when special 'rescue funds' run out at the end of the year.

The provision of the reunification treaty that academy institutes should either be closed down or given another status meant that much of the research at Adlashof was taken over piecemeal by the four main pillars of German research — the Max Planck Society, the Fraunhofer Society (for applied research), the national research centres and 'Blue List' institutes (which are equally funded by the federal and *Länder* governments). Industry also showed some interest, while several groups were given grants to set up small businesses.

But somehow the nucleus of chemical research at Adlashof, although favourably evaluated, fell through the net. Although four institutes (for inorganic polymer chemistry, heterogeneous catalysis, macromolecular chemistry and organic synthesis) were graded as excellent, the Wissenschaftsrat's 1991 proposals for their continuing support have fallen apart.

The Wissenschaftsrat recommended that staff numbers should fall from 1,700 to 325. At this stage, it was idealistically aiming to reintegrate research into the universities from which it had been all but banished 20 years earlier. It also recommended that, over a period of seven years, the institutes should either form an outpost of the Humboldt University in Berlin or separately move to other universities in the east.

Events have shown that the universities

Dorian Gray mice exposed

SIR — Congratulations on the wonderful April fool's story by Robin A. Weiss on Dorian Gray mice (*Nature* **362**, 411; 1993). I have assigned it to my graduate students for review and opinions. I wonder what they will make of it. I also left it on the desk of a young colleague who directs a vector core facility and designs retroviral vectors, with the request to put the *tith* gene under various tissue specific promoters so that we may keep certain parts of male or female anatomy forever young.

Olivera J. Finn

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SIR — As a regular reader of *Nature* in a non-British country with a less-developed sense of humour, and admitting that I may be at the negative end of the fun spectrum, I suggest that the article "Dorian Gray mice" will not only provoke smiles from biologists but may have more severe consequences. Indeed, in Germany there is in reality a group calling itself the "Genethisches Netzwerk" which tries to swamp the German population with accusations not very different from that of preprogrammed death.

Nature is known as a scientific publication and not as a magazine for the entertainment of its readers. Also, I am afraid that among serious people the distinction between fact and fiction will be blurred. *Nature* is read by many journalists who may not be able to distinguish between real and fictional data and may therefore be misled.

Peter Starlinger

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SIR — We read with interest the article on Dorian Gray mice. As froggies, we appreciate the fascinating possibilities of increasing the size of these charming little animals. But the paper by Ariellos *et al.* was misquoted. In fact, it is notorious that this marvellous work was edited by Chatqui-expire, the famous scholar of the soul in one of its best monographs, *The Tempest*.

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SIR — As usual in the field of molecular biology, the origins of experiments are forgotten. Weiss's masterly exposition of ageless mice research has forgotten my own signal contributions. After many a summer in Messina, I not only discovered phagocytosis but I also formulated the proposition that ageing was due to the interaction of phagocytes with intestinal bacterial. My swan song — so to speak — was to propose the antidote: yoghurt¹.

Elie Metchnikoff

As communicated to

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1. Metchnikoff, E. Z. *Dannon Res.* **3**, 1143 (1992).

SIR — As apoptosis is a common mechanism of the death of T cells, perhaps Robin Weiss's article should have been entitled "Earl Grey mice".

John Moore

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SIR — Weiss's article about immortal transgenic mice caused great interest among staff and students at this university. Several people were seen looking frantically for more details on the subject for their next seminars, while others enthusiastically held forth to their students on this new and exciting matter. Still others were already trying to get cDNA probes for longevin and tithonin, with the intention of performing widespread screening of all kinds of libraries. In addition, at least two grant applications were hastily submitted in the first week of April. This was not due to naivety on the part of Spanish researchers and students — the equivalent in Spain of April fool's day is Innocents' Day on 28 December. So we were caught by surprise as we do not expect this kind of joke to be played in spring. In the end we all had a good laugh because it was a joke, right?

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■ The book review by Orlando Belpaese on page 422 was also an April Fool's joke — Editor, *Nature*.

Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

Dating ice cores

SIR — The data obtained on ice cores from the European (GRIP) and the US (GISP2) drilling projects are of great interest to the palaeoclimatic community. The first papers regarding timescales for the past 40,000 years have been published in *Nature*¹⁻³. The main purpose of this correspondence is to convey to the scientific community that a shared effort now exists to compare and refine the dating of these cores². The timescales were constructed by each group independently by counting successive annual layers by various methods. The dates of the two cores back to 14,500 calendar yrs BP agree exceptionally well within expected error (250 years) where counting was continuous ($\Delta T = 230$ years) at the start of the Bølling (14.5 kyr ago). Below this, spot counting and interpolation leads to divergence in the timescales. This deviation increases progressively to 40 kyr where the difference approaches 5 kyr. However, the scales still remain consistent within estimated errors (± 3.5 kyr). At a recent GRIP/GISP2 workshop, the US and European scientists responsible for the dating in the two projects exchanged information regarding the age dating of the cores leading to the following:

(1) The present difference between the timescales is essentially due to the preliminary character of the work. The data were published before complete analysis of the age-depth relationship because of their importance to the scientific community.

(2) A joint GISP2/GRIP paper on dating to 40 kyr BP is to be finalized during a common workshop and it is the intention of those involved that a joint paper on the dating of the entire record back to at least 140 kyr will be completed.

As chairpersons of the GISP2/GRIP dating committees, we stress the importance of the independent dating of the ice cores as it is a prerequisite for the meaningful comparison with other palaeorecords in a truly consistent global way. These relationships will allow us to develop our understanding of global climate change and of the climate forcing parameters that drive it.

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1. Johnsen, S. *et al.* *Nature* **359**, 311–313 (1992).

2. Taylor, K. *et al.* *Nature* **361**, 432–436 (1993).

3. Alley, R. *et al.* *Nature* **362**, 527–529 (1993).

Competition and the death of science

It is natural that research should be competitive, but there is now ample evidence that competition is too fierce for many people's peace of mind and for the health of the scientific enterprise as a whole.

COMPETITIVENESS in science has done wonders for both the quantity and the quality of discovery in the past few decades. Of the quantity, there can be no doubt; familiar statistics of the steady growth of the literature, of the proportions of deserving grant applications that grant-giving agencies cannot support and of the demand for space in reputable journals are ample proof that the river of discovery is in flood. Quality, most easily assessed with hindsight, is also steadily improving in at least one important sense: competitiveness ensures that people take immense care not to make too much of the data they have gathered for fear, afterwards, of seeming fools. That makes the literature duller than it needs to be, but that may be a virtuous fault.

Nor is it surprising that competition should be common even in science. The edifice of discovery may be built from abstract ideas, but the builders are only human. Most probably, the present competition for research grants, laboratory space, the brightest graduate students and so on is merely an extrapolation into modern circumstances of the *primaeval* instinct to compete for edible resources. If the outcome is the marvellously deepening understanding of what the world is like that we now enjoy, who, except perhaps the spouses of the postdoctoral fellows whose laboratory lights burn all the night, can reasonably complain?

Of course, this coin has another side. There is little doubt that the competition to succeed in discovery, or at least to be seen to succeed, explains the rash of perversions of the literature in the past 15 years or so. Outright fraud may be comparatively rare, the magnification of achievement by making thinner slices of one gobbet of discovery is not. Nor is the deliberate misreferencing of other people's work in a manner that magnifies the importance of one's own. Most people know the dangers, many have suffered from them.

The side-effects of competitiveness are not simply a contemporary phenomenon, of course. The great clash between the newtonian and cartesian schools lasted for much of the eighteenth century. The foundation of the atomic theory in the early decades of the following century was not free from skulduggery. But by the early decades of this century, it seems to have been implicitly recognized that outright expressions of competitiveness may damage not only people but even the progress of science itself. That is when it became estab-

lished that people with discoveries to boast of should answer serious enquiries about them, explaining even to their competitors exactly how they had succeeded. What follows is mostly a lament for the passing of those genteel decades, based mostly on this journal's recent and often distressing experience.

The reasons why good manners have recently been debased are readily understood, and have much (not all) to do with the publication process. In the old days, when the scientific enterprise was much smaller, people's achievements, while embodied in publications, made their reputations mostly by word of mouth. Neither Maxwell nor J. Willard Gibbs would have felt compelled to publish something every other month. But now, when a publication record is almost the sole determinant of reputation and thus, by way of research grants and promotions, of success and self-esteem, competitiveness centres uncomfortably on the journals.

Journals (even this one) abet competitiveness, often willingly but sometimes unwittingly. In essence, they compete among themselves (by means of the service they offer contributors) for what they consider to be the most interesting articles and then, by their decisions, endow what they publish with a degree of importance not necessarily commensurate with its intrinsic worth. Increasingly, the competitiveness of journals and authors centres on the time and even the timing of publications.

Here is an example of what can happen. On 13 November last, Dr Manuel Perucho and his colleagues at the California Institute of Biology at San Diego offered *Nature* a manuscript describing the deletion of nucleotides in tracts composed of alternating A and T residues from the genomes of cells in a subset of human colon carcinomas. Afterwards, it emerged that essentially the same manuscript had been seen and refused by two other reputable journals. In the usual way, the manuscript was sent to two referees (avoiding at the authors' request a noted expert in the field). Each expressed enthusiasm, but at the same time suggested important ways in which the findings could be strengthened. The manuscript was therefore returned on 12 January with a note saying we would be happy to consider a resubmission if the authors were so inclined. (They were, and another version, including data on other kinds of repeats and a range of tumour stages, arrived a month later.) By mid-

March, after further review and some drastic pruning, Perucho was told that his paper would be published.

Then came a bombshell of sorts. On 7 May, three articles announcing essentially the same result appeared in the journal *Science* (Peltomäki, P. *et al.* **260**, 810; Aaltonen, L.A. *et al.* **260**, 812; and Thibodeau, S.N. *et al.* **260**, 816). The first two articles are recorded as having been received on 8 April, and were accepted for publication within the week; one of the senior authors of the first two articles was Perucho's excluded referee. The third article, equally interesting, had originally been submitted a month earlier. In mid-April, Perucho was telephoned by *Science*, seeking information about his work for a general article on the three articles, each of which (from internal evidence) must have entailed several years of work. Perucho's article appeared in *Nature* on 10 June (and will repay reading).

No blame attaches to *Science* or the authors of these papers for having rushed them into print; given the chance, *Nature* would probably have done the same. But there are three casualties. First, Perucho, who feels he has been robbed of the glory that was rightly his. Second, the interest of the discovery has been diminished by all the fuss; the cancers whose cells carry shortened repeats are differently distributed in the colon from others, and metastasize less frequently, for example, while if Petrucho is right in believing that the underlying fault may be a mutation of a DNA repair gene, the ramifications of that may be exceedingly important.

The third casualty is science itself. So much zeal is now invested in being first, and so much emotion is spent if seeming to be second, that people's effectiveness must be undermined. But that is the least of the harm done. Will Perucho in future be as open in talking about his work at meetings as he has apparently been? Will his younger colleagues not regard his experience as a proof that they must be even more zealous competitors, secretive until their discoveries are published, then ruthless in winning recognition for them?

The only lasting remedy is that the link between publication records and reputations should somehow be broken. Academic institutions have the chief responsibility. Journals have also an important part to play. If good manners are entirely banished by competitiveness, neither will have a long future.

John Maddox

Supernovae can't be typecast

Paul Murdin

THE once convenient classification of supernovae is coming under increasing pressure as observational techniques put it to more and more severe tests. Indeed, it seemed at a colloquium held in Xian last month* that the recent supernova, SN1993J, the brightest in the northern skies for more than 50 years, has transgressed the boundary between types within just weeks of appearing.

Astronomers have developed a simple code dividing the appearance of the optical spectra of supernovae into types I and II. If the spectra of type II supernovae look unusual, it is because their physics is so unusual, with the conditions completely out of equilibrium (whereas stars remain in equilibrium for millions of years); in fact their composition is normal, like that of the Sun.

A type II supernova arises from the gravitational collapse of the iron core that nuclear fusion has built in the centre of a massive star. The collapse energy gives the explosion that drives off the outer layers of the star (typically 10 solar

masses), which have been little processed by nuclear fusion, and so have a fundamentally normal stellar composition. SN1993J, which erupted in late March, was a type II supernova, but the outer layers of its progenitor star were of low mass and are very quickly becoming transparent as the supernova expands — already it is demonstrating the weakness of over-simple classification. Light output from the supernova at maximum on 31 March (Fig. 1) was weaker than usual and the light intensity died away unusually quickly from maximum. The amount of ejected material was only 2.5 solar masses, far short of the 'typical' value of 10 solar masses; and only 10 per cent of this was hydrogen and helium (S. Woosley, Univ. California, Santa Cruz; K. Nomoto, Univ. Tokyo).

A second peak in the intensity on about 20 April represented the arrival at the surface, after radial diffusion, of energy derived from the radioactive decay of 0.06 solar masses of ^{56}Ni created by nucleosynthesis in the explosion. ^{56}Co is the product of the decay of ^{56}Ni , itself radioactive, and the curve of total radiated power from SN1993J is now showing the characteristic exponential decay rate from this isotope.

SN1993J is only the second supernova (after SN1987A) for which the progenitor star has been identified. The progenitor, a K-type supergiant, is likely to have originally had much more mass than the 4 solar masses that astronomers can account for in SN1993J (2.5 solar masses of ejecta, 1.5 solar masses in a hypothetical neutron star). Some of this missing material revealed itself in early spectra of SN1993J in the form of circumstellar coronal emission lines of highly ionized iron, observed from the ground (P. Meikle, Royal Green-

wich Observatory; T. Matheson, Berkeley), and nitrogen observed by the International Ultraviolet Explorer satellite (R. Kirshner, Harvard-Smithsonian Center for Astrophysics). These ions were excited by the explosive shock as it broke out from the surface of the supernova.

The interaction between the expanding supernova and the circumstellar environment caused increasing radio emission from SN1993J, observed by the Ryle Telescope (D. Green, Mullard Radio Astronomy Observatory) and by the Very Large Array (S. van Dyke, VLA). Strong shocks between ejecta and circumstellar material also produced soft X-ray emis-

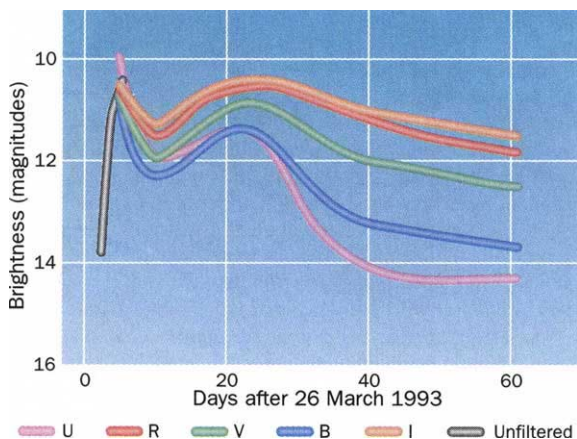


FIG. 1 Light curves (brightness variations) in several wavebands for SN1993J, based on observations from La Palma. Ultraviolet, U; blue, B; visual, V; infrared, I; and unfiltered. Brightness given in the logarithmic magnitude scale.

masses), which have been little processed by nuclear fusion, and so have a fundamentally normal stellar composition.

The composition of type I supernovae is, on the other hand, completely abnormal. There are two main subclasses. Type Ia events develop iron and cobalt emission; and type Ib supernovae have helium absorption lines at first and subsequently develop oxygen and calcium emission. These compositions are typical of material that has been processed to different stages of the nuclear cycle ($\text{H} \rightarrow \text{He} \rightarrow \text{C} \rightarrow \text{O} \rightarrow \dots \rightarrow \text{Fe}$).

*Supernovae and Supernova Remnants, IAU Colloq. 145, Xian, China, 24–29 May 1993.



FIG. 2 SN1993J, on the edge of the spiral galaxy M81, outshines foreground stars; photographed on 4 April by R. Willstrop.

sion detected by Rosat only six days after the explosion (the earliest that X-rays have been seen from a supernova). Asca ('Flying Bird'), a new Japanese satellite that was launched on 20 February, also detected these X-rays and observed SN1993J during its performance verification phase. Up to 15 May, the X-ray intensity of SN1993J was fading little by little (H. Tsunemi, Univ. Osaka).

How the progenitor of SN1993J lost so much of its material remains controversial. An American 'school' believes that its outer layers were stripped off by a companion star. However, an isolated star of 20–30 solar masses could lose well over half of its mass through stellar winds (P. Höflich, Univ. Munich).

Because the progenitor of SN1993J had lost so much of its outer material, expansion of the sparse remaining hydrogen layer in the supernova quickly revealed the helium shell within. Thus several groups (A. Filippenko, Univ. California, Berkeley; Meikle) showed that the type II spectrum of SN1993J had developed unusually strong helium absorption by the time of the colloquium, where participants generally agreed that SN1993J was becoming a type Ib. This subclass of supernovae has the same explosive mechanism as type II but takes place inside a star that has entirely lost its hydrogen envelope. Models for SN1993J predict (Nomoto; Woosley) that hydrogen and helium will be visible for a few months, but that soon SN1993J is likely to look like a pure type Ib (C. Wheeler, Univ. Texas). The one previous case of a type II metamorphosing into a type Ib was SN1987K (Filippenko), which was incompletely studied as it was behind the Sun at the time of change. With its metamorphosis heralded, SN1993J is sure to be closely monitored. The changing apparent composition will be a crucial test of models of the progenitor star and how it came to be the way it was.

By contrast to types II and Ib, type Ia supernovae have been regarded as very uniform. On the conjecture that a type Ia

represents the collapse of a carbon-oxygen white dwarf star at its limiting Chandrasekhar mass (1.5 solar masses), and so can behave in only one way, type Ia supernovae have been proposed as reliable, bright standard candles by which to measure the scale of the Universe. The participants' confidence in this conjecture was severely shaken by the developing body of evidence of irregularities in type Ia behaviour.

Besides smaller differences in the expansion velocity of the supernova ejecta and spectral features, there are important differences in the light curves. N. Suntzeff (Cerro Tololo Inter-American Observatory) showed that SN1991bg was up to ten times fainter than the canonical type Ia supernova. Accurate tracking of the light (B. Leibundgut, Univ. California, Berkeley) from other type Ia supernovae (including SN1992A), shows differences in the brightness of the long decay tail. As in type IIs, this decaying luminosity is powered by the radioactive decay of ^{56}Co ; from the light curves and from theoretical fits to spectra (P. Lapuente, Harvard-Smithsonian Center for Astrophysics; P. Mazzali, Osservatorio Astr. di Trieste) it seems that varying amounts of the isotope are produced.

What, asked R. Canal (Univ. Barcelona), could produce such a wide range in the light curves, but at the same time usually select an intermediate value? One possibility is the collapse of a white dwarf whose mass is different from the Chandrasekhar mass. If material is quietly dribbled onto a white dwarf from outside, for example from a companion red giant star (A. Renzini, Univ. Bologna), one might imagine a white dwarf type Ia progenitor always building up to the Chandrasekhar mass, at which point it would give the standard type I explosion. But if two sub-Chandrasekhar white dwarfs merged from a close orbit, the resulting single white dwarf might itself be sub-Chandrasekhar, yet be detonated by shocks in the merger process. Other possible internal factors that could confound the conventional picture include stellar vibrations that might trigger detonations in sub-Chandrasekhar white dwarfs, and off-centre detonations.

Wheeler tried to restore confidence somewhat by revealing that a lunch-time conclave had concluded that "there does remain a standard type Ia", represented by, among others, SN1972E, 1981B and 1989B. So if abnormal detonations are rare, type Ia supernovae may remain good cosmological candles, but not, evidently, entirely standard ones; the possibility of systematic differences over the cosmological timescales that are being probed would be additionally worrying. □

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Transposons hop out of maize

Robert Shields

TRANSPOSON tagging in a heterologous (foreign) plant host can be made to work. That news, long-awaited, means that another and effective route to identify plant genes of agricultural or scientific value is now open. Transposons are mobile pieces of DNA present in many (if not most) living organisms, but they were first discovered in maize in 1948 through the pioneering work of Barbara McClintock. A transposon landing in a gene acts simultaneously as a mutagen and a gene tag, and numbers of genes from

been turning up lots of genes of great interest^{2,3}. However a small flurry of papers, including one on page 715 of this issue⁴, now shows that heterologous tagging is not a mildly eccentric hobby of a band of enthusiasts but a technique capable of producing results of real value.

Although the different laboratories involved used different transposon systems to tag different genes, the approaches are markedly similar. A vector carrying the transposon is introduced into the plant

genome via *Agrobacterium* transformation. Once the transposon excises it preferentially re-inserts in non-repetitive DNA so there is a fair chance of it dropping in a gene and causing a mutation⁵. If the excision and re-insertion occur in cells that go on to form the gametes, then cycles of self-pollination will produce mutant plants homozygous for the transposed element and the tagged recessive gene. Re-excision from the mutated gene can restore gene function, which results in a patch of revertant cells in an otherwise mutant plant.

Reversion is the vital clue implicating a transposon as the cause of the mutation. A report of the tagging of the *Ph6* gene in petunia was the first to emerge, the success of the experiment revealing itself in the

flower through patches of dark purple (wild type) on a pale (mutant) background (see figure)⁶. In the case of the male sterility gene described by Aarts and colleagues in this issue⁴, revertant cells in the stem of an otherwise male sterile plant gave side branches heterozygous for the mutation and sporting fertile flowers; likewise, a tagged albino mutant gave revertant green sectors (G. Coupland, personal communication) and a severe morphological mutant reverted to normal⁷.

Transformation with *Agrobacterium* introduces only a few copies of transposons into plants, so it is possible to identify the element causing the mutation by segregation analysis and clone the mutant gene using the transposon as a probe. In contrast, plant transposons in their natural



Tell-tale flower — a variegated petunia corolla produced by a plant in which the *Activator* transposable element from maize had been inserted into the *Ph6* gene. Mutations in *Ph6* alter acidity, and therefore anthocyanin pigmentation. The variegation seen here results from excisions of *Activator* during flower development which restore *Ph6* function. (Reproduced from ref. 6.)

numerous organisms have been isolated in this way. But until now transposon tagging in plants has been limited to species whose own transposons had been well characterized.

Nearly seven years ago, the maize transposable DNA element *Activator* was introduced into tobacco and shown to move to new sites in the genome¹. Reports of similar findings in other plants rapidly followed, leading to the enticing prospect of tagging genes in plants where no transposons had been identified. Since then the failure to tag a single gene by this approach has been something of an embarrassment — doubly so in fact, for less glamorous methods such as tagging with the T-DNA of *Agrobacterium tumefaciens* or positional cloning have

Am. Soc. Plant Physiologists

habitat can exist in several dozen copies, complicating analysis and cloning. Molecular analysis of revertants confirms that the tagged sequence causes the mutation without having to conduct tedious complementation experiments.

Analysis of mutant and revertant alleles shows that transposon excision rarely restores the gene to its original shape; typically, a 'footprint' of extra nucleotides is left behind. If insertion happens in a coding region (as it did with the male sterility and morphological mutants), reversion is accompanied by restoration of the protein-reading frame. However the albino mutant reverted even though reading frame was not restored, suggesting the insertion was not in an exon. In other plants imprecise excision of transposons from promoters or untranslated regions has produced alleles of genes with new and intriguing patterns of expression^{8,9}, and it is likely that the same will be true for heterologous transposons.

All the mutants identified were picked out because the phenotype they displayed was quite dramatic. Many interesting genes do not have such an obvious effect (those responsible for disease resistance, for example). Is there any way that these might be identified? If allelic variants exist then it is possible to place these genes on a genetic map. In theory it would then be possible to walk (or more likely trudge) towards the gene from flanking restriction fragment length polymorphism (RFLP) markers. It is then a question of working out which of the genes that are found are responsible for the phenotype. The method has proved spectacularly successful in identifying genes responsible for genetic defects in humans, and there are several examples of it working in plants³.

Heterologous transposition offers a new alternative. As *Agrobacterium* introduces DNA at random sites in the genome, plants could be selected where the start site for transposition is near the gene of interest. As some transposons show a marked preference to transpose locally¹⁰, it should be possible to pepper a restricted region of the genome with transposon insertions. If the insertion knocks out the targeted gene it could then be

cloned by the methods outlined above.

So why has it taken so long to tag genes with a heterologous transposon? Part of the answer is that much of the development work was done in tobacco, which is effectively tetraploid for many genes making tagging of recessive alleles difficult (although it may have been achieved for the *sulphur* locus; W. P. Fitzmaurice, personal communication). Also it was not appreciated that one of the steps — the tissue-culture stage — necessary for transformation can produce as many mutations as an active transposon¹¹, and that transposons can insert and excise rapidly leaving a mutant footprint with no tag. It is also now apparent that many plants thought to lack endogenous transposable

elements do in fact have them and that they can produce a significant background of unstable mutations¹². It may also be that, as in yeast and *Drosophila*, many genes can undergo mutation without giving a phenotype^{13,14}.

But the take-home message from the new work is a more general one. Just as the study of transposons in a heterologous host has led to a rediscovery of much of the classical work with these elements, molecular biologists have rediscovered that plant genetics is hard work and takes time. □

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IMPACT CRATERS

When will enough be enough?

Richard A. F. Grieve

MJØLNIR, a 40-kilometre circular feature beneath the waters of the Barents Sea, may be the latest in the growing list of terrestrial impact craters. In a recent edition of *Geology*¹, S. T. Gudlaugsson reports on the discovery of a structurally disturbed area within Jurassic–Cretaceous sediments on the continental shelf off the north shore of Norway, and argues that it was created by an extraterrestrial body perhaps 2 kilometres in diameter something like 140 million years ago.

The structure is defined by a series of geophysical anomalies. Seismic reflection data reveal the form of a complex impact structure. Its gravity and magnetic signatures are also consistent with those over known, large impact structures. Gudlaugsson considers alternative origins — for example, that it is a salt or shale diapir or a volcanic intrusion — but rules them out for a number of geological and geophysical reasons. Of course, Mjølñir has not been drilled, so that no geological samples are yet available. Until it has, and until evidence is found of shock metamorphic effects in the target rocks, diagnostic of the extreme impact pressures, Mjølñir will be strictly speaking only a probable impact structure.

In recent years, more and more terrestrial impact structures are being discovered. Last year, two hydrocarbon-bearing impact craters were discovered in the United States: the 16-kilometre Ames structure² in Kansas and the 12-kilometre Avak structure³ in Alaska. Several other new findings have been reported from other regions of the world. This apparently accelerated rate of discovery begs the question "why now?". There are undoubtedly a number of reasons. Amongst them is the greatly heightened awareness of the occurrence of impact events on

Earth brought about by the high-profile debate over the Cretaceous/Tertiary (K/T) boundary, the extinction of many species and its relation to a major impact event 65 million years ago. Another reason is probably the increasing maturity of our knowledge of the geological and geophysical effects of impact. The characteristics of impact craters are no longer known to only a small number of specialists. Indeed, most of the recent discoveries of terrestrial impact craters have been made by members of the larger earth-science discipline. Like the shatter cones at the Sudbury structure in Canada, which went unnoticed by geologists for decades, the record of impact is only now becoming more apparent because the community knows what to look for and how to interpret the evidence.

Do all these new discoveries require a reassessment of how often such impact events occur? The answer is no. For example, if Mjølñir is an impact crater with a diameter of about 40 kilometres and an age of 140 ± 20 million years, then it joins a relatively short list of similar or larger craters. There are only seven known craters, including Mjølñir, with diameters greater than 40 kilometres and ages less than 160 million years. From present estimates of the terrestrial cratering rate⁴, around 30 such structures are expected to have been formed on the Earth's land surface over this time.

In addition to impact craters, the signatures of impact are beginning to be recognized in the stratigraphic record. For example, K. Wang⁵ recently described what appear to be microtektites in upper Devonian limestones, which may be the record of a large impact event 360 million years ago. By far the most publicized evidence for impact in the stratigraphic

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RÉSUMÉ

Against addiction

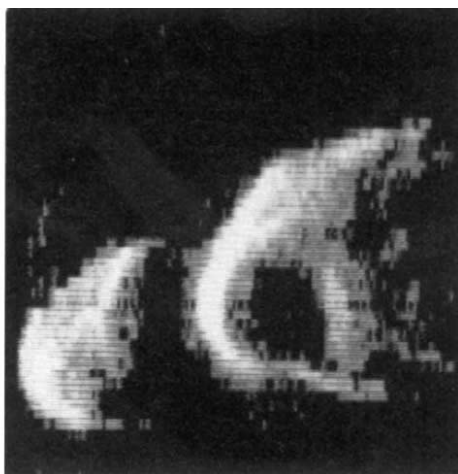
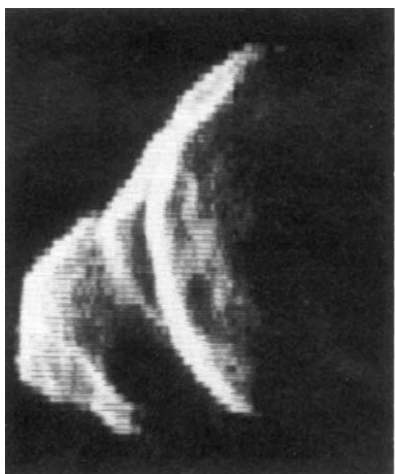
PICKING their way through the tangled thicket of dopamine receptor subtypes, S. B. Caine and G. F. Koob have alighted on the D3 form as a promising point of attack on cocaine addiction (*Science* **260**, 1814–1816; 1993). Dopamine, a neuromodulator, seems to be at the centre of brain pathways that mediate responses to certain drugs and is also implicated in schizophrenia. The receptors through which it acts are still being characterized. But Caine and Koob demonstrate that the affinity of binding of three agonists to the D3 form correlates very well with the effectiveness of the agonists in reducing self-administration of cocaine by trained laboratory rats. Crucially, two of these agents — 7-OHDPAT and quinpirole — reduced the rats' craving for cocaine at doses of agonist that themselves seemed not to be addictive.

Flare up

A STELLAR X-ray flare that eclipses all others has been observed by the X-ray satellite Rosat (M. A. Smith *et al. Astrophys. J.* **409**, L49–L52 (1993)). With an output of 4×10^{18} megawatts (the total output of the Sun is only 100 times larger) and a duration of 14 hours, the flare put out 100 times more energy than its closest rival. The astronomers were looking at the star Eridani, a member of a class of stars, termed Be types, that undergo sporadic episodes of mass loss. The hope had been that these stars' X-ray activity might reveal the mechanism responsible for mass loss, but until now the results had been pretty unimpressive. The detection of this flare, however, seems to implicate violent magnetic behaviour like that responsible for solar flares, but writ large.

Temperature switch

SALMON fishermen should arm themselves with a thermometer, as well as rod and line, if the results of laboratory experiments carried out by N.H.C. Fraser, N.B. Metcalfe and J. E. Thorpe apply in the wild (*Proc. R. Soc. B* **252**, 135–139; 1993). The authors looked at the activity and feeding patterns of young salmon under controlled lighting conditions and variable temperature, and found that there is a dramatic switch in behaviour at 10 °C — above that temperature the fish feed largely by day, below it largely by night. This change in behaviour, they believe, is associated with changes in the salmon visual pigments at lower temperatures which confer better night vision. And they speculate that the reason for the switch lies in a delicate balance between feeding efficiency and risk from predators in winter and in summer.



Dynamic duo — asteroid 4179 Toutatis, in radar images obtained by S. Ostro at the Goldstone Deep Space Communications Complex in California on 8 and 9 December 1992, when the object was just ten times further from the Earth than is the Moon. Like other near-Earth asteroids observed by Ostro and collaborators, Toutatis is composed of two distinct objects (in this case 2.5 and 1.5 km across) in contact with one another.

record is, of course, at the K/T boundary. While still debated, it would seem that some of the controversy has subsided, with the linking of global stratigraphic evidence of the boundary's to the Chicxulub structure^{6,7} in the Yucatan, Mexico. Chicxulub, like Mjølner and other recent discoveries, is buried and was initially discovered through geophysical anomalies. Although some palaeontologists and geologists are unwilling to subscribe to a cause and effect relationship with respect to the mass extinction at the K/T boundary, it is becoming harder and harder to deny that a massive impact did take place.

The evidence for the 200-km diameter Chicxulub impact crater being the cause of the K/T boundary event is formidable; nevertheless other candidate craters — at Manson, Kara, Kamensk and Gusev — have been put forward. This has led to suggestions that the K/T event was, in fact, a series of impact events produced by a number of comets, perhaps due to the break-up of a large comet as was observed recently by C. and E. Shoemaker and D. Levy (see the 10 June issue⁸). The notion that all these craters are of exactly K/T boundary age is based on a relatively poor set of age estimates for some of them. Chicxulub is clearly the candidate of choice for producing effects in the biosphere.

As more sophisticated analyses are performed on K/T boundary materials and the appropriate crater lithologies, I believe that impact structures such as Manson will be shown to have little or nothing to do with the K/T boundary event. They represent simply the background of impact cratering against which the truly massive events such as Chicxulub occur. Not that simultaneous impact events do not occur. There are examples, such as the East and West Clearwater structures, which require the impact of contact binary

bodies, similar to the twin-lobed asteroids recently imaged by S. Ostro using radar (see figure).

There is mineralogical and geochemical evidence from spinels in the K/T boundary layer that is contributing to the debate over multiple impacts. Indeed, E. Robin, L. Froget, C. Jéhanno and R. Rocchia, in last week's *Nature*⁹, report on the occurrence of high Ni and Fe³⁺ spinels at the K/T boundary in a deep-sea core from the Pacific. The compositional data are interpreted as indicating that these spinels are meteoritic ablation debris. As they are located 10,000 km from Chicxulub, Robin *et al.* interpret this as a separate but simultaneous event. They suggest that the spinels are due to the impact of a 2-kilometre asteroid, about the same size as the body that would have produced the Mjølner structure, and they argue that several such events are needed to explain the global distribution and the character of the spinel-bearing spherules at the K/T boundary. Much is still to be learned, particularly with respect to what occurs in the atmosphere during large impact events. Whether this interpretation of the spinel data is a strong constraint for multiple events at or near the K/T boundary remains to be seen. □

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Efficiency in activation

Steven Hahn

TRANSCRIPTION factor IIB is one of the main cogs in the machinery through which DNA is transcribed into messenger RNA by the enzyme RNA polymerase II. The molecule is of intense interest, in part because it is implicated as a target of so-called activator proteins which stimulate the process of transcription. Slowly, it is yielding its secrets — a new crop of papers¹⁻⁴, including one on page 744 of this issue¹, tells us more about the TFIIB regions essential in basal transcription, while on page 741 a further report⁵ provides strong support for the idea that acidic activators act in part by coming into contact with TFIIB.

Along with the general transcription factors TFIID–TFIIF, TFIIB is essential for precise positioning of RNA polymerase II (pol II) at promoters. After initial binding of TFIID to the TATA box, TFIIB is brought to the complex through protein–protein interactions with the TATA binding protein (TBP). In association with TFIIF, pol II next binds to the preinitiation complex. TFIIB is thought of as a bridging factor because it uses protein–protein contacts to bind both TBP and the pol II–TFIIF complex^{2,6}.

It seems that the function of TFIIB in transcription was set early in evolution, as a TFIIB-related factor known as BRF1 is essential for transcription by RNA polymerase III (pol III), which catalyses the synthesis of transfer RNA; BRF1 and TBP, and at least one other factor, comprise TFIIB⁷. In light of the similarity in sequence between BRF1 and TFIIB

features. First, at the amino terminus there is a conserved cysteine-rich sequence postulated to be a zinc-binding region. Second, two imperfect repeats of about 75 amino acids comprise most of the carboxy-terminal sequence. Finally, a cluster of basic residues at the end of the most amino-terminal repeat are spaced such that they could potentially form an amphipathic α -helix.

In the new papers, the amino- and carboxy-terminal regions are defined as separate domains with distinct functions. Digestion of TFIIB with trypsin cleaves at residue 105, leaving a protease-resistant carboxy-terminal core (ref. 3; R. Roeder, personal communication) which is probably in a tightly folded domain. The amino-terminal region is degraded, suggesting that it is in a more extended or flexible conformation. The carboxy-terminal core functions in binding to the TBP–DNA complex but is inactive in transcription. These results are in agreement with extensive deletion analysis of TFIIB¹⁻⁴. Deletion of any part of the carboxy-terminal core abolishes binding to the TBP–DNA complex, and deletion of the putative zinc-binding domain cripples

activity in transcription. This latter transcription defect is due to the failure to incorporate pol II–TFIIF into the preinitiation complex. So the amino-terminal TFIIB region is probably involved in protein–protein interactions with pol II, TFIIF or both.

What, though, are the targets and mechanisms of transcriptional activators? Although it is presumed that activators touch a component of the general transcription machinery and facilitate some step in initiation, the exact mechanism is unknown. By perhaps a second mechanism, activators are thought to relieve repression by chromatin-associated proteins (for review see ref. 9). Analyses with affinity chromatography have pointed to both TBP and TFIIB as potential targets of acidic activation domains, such as that found in the herpes simplex virion protein VP16 — the VP16 activation region can selectively interact with TBP¹⁰, and with TFIIB¹¹; and under some conditions VP16 must be present during incorporation of

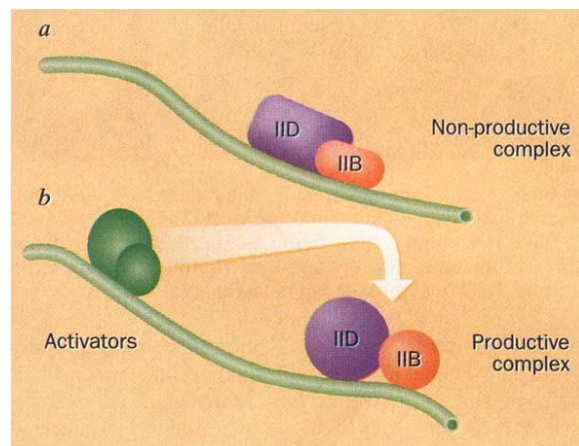


FIG. 2 Model for the function of acidic activators. *a*, In the absence of activator, TFIID and TFIIB assemble to form a non-productive complex. This complex may bind pol II and other factors but initiate transcription with low efficiency. *b*, In the presence of activator, TFIID and TFIIB assemble to form a productive complex. After assembly with the other factors, polymerase initiates transcription with high efficiency.

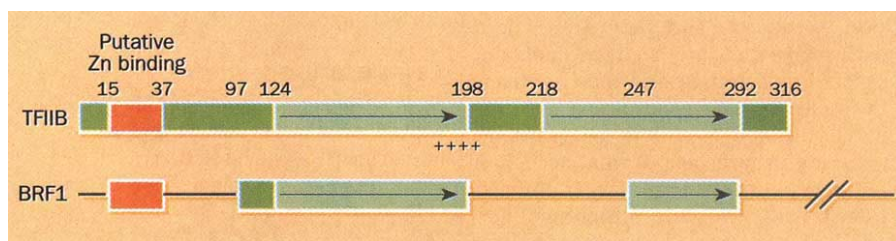


FIG. 1 TFIIB and the related pol III factor BRF1. Numbers refer to amino-acid residues in human TFIIB. The three most highly conserved regions between BRF1 and TFIIB¹⁵ are also shown.

(Fig. 1), the two factors probably have much the same function in contacting polymerase and TBP. A putative TFIIB homologue has also been found in archaeobacteria⁸. Given the similarity between the machinery for the three nuclear polymerases, it would not be surprising if a TFIIB-like factor is also required for transcription by RNA polymerase I (pol I), which synthesizes ribosomal RNA. All of this goes to show just why TFIIB has attracted so much attention.

The sequence of TFIIB from humans and other organisms has several notable

TFIIB into the preinitiation complex to allow high levels of transcription¹¹.

If these protein–protein interactions are important in activation, (1) at least some VP16 activation mutants should be defective in binding to TBP and TFIIB, and (2) some mutations in TBP and TFIIB that fail to bind the VP16 activator should function normally for basal but not activated transcription. The first prediction has been borne out for both TBP and TFIIB; there are indeed VP16 activation mutations that reduce binding to both general factors^{12,13}.

The second prediction has now been met for TFIIB. Roberts *et al.*⁵ defined the putative amphipathic helix in TFIIB as one of two regions necessary for interaction with the VP16 activator. Mutagenesis of TFIIB in this region by changing residues from basic to acidic identified two double mutations that fail to bind VP16, as measured by affinity chromatography. Both of these TFIIB double mutations still function in basal transcription but fail to respond to activation. These results constitute strong evidence that there is a functional interaction between TFIIB and acidic activators, but they do not mean that TBP is not also a target; several activators are usually necessary for maximal activation, so it is certainly possible that activators have more than one target.

What could activators do to facilitate initiation? A popular model is that acidic activators facilitate the binding of the general factors TBP and/or TFIIB to the promoter, but it has little direct support and is in fact contradicted by much experimental data. In direct DNA-binding studies, TBP, TFIIB and the rest of the transcription machinery bind with reasonable affinity to DNA in the absence of activators. However, the success rate of transcription initiation is likely to be very low, with only a fraction of promoter templates used.

An alternative model is that activators increase the efficiency of polymerase initiation by interaction with TFIID, TFIIB or both⁹. Recall that activator, TFIID and TFIIB are all required during assembly of the preinitiation complex to achieve high levels of transcription¹¹. In the absence of activator, TFIID and TFIIB might assemble non-productively to form inactive initiation complexes^{9,14} (Fig. 2); this might be due to inherent properties in the proteins themselves and/or to factors binding to TFIID-TFIIB and inhibiting further assembly of the complex. In the absence of transcription inhibitors, these non-productive complexes may bind polymerase and the remaining general factors, but RNA synthesis would occur from only a fraction of them. In the presence of activator, TFIID-TFIIB would assemble in a productive manner giving a high percentage of active initiation complexes. If so, one would predict the existence of mutations in TFIIB or TFIID which would shift the equilibrium towards either the productive or the non-productive complexes. But whatever the mechanism for activation, it seems certain that identification of the target is only the first step in a detailed understanding of the mechanism of gene control. □

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Not just a lot of hot air

Mark Chandler

THE 1980s were warm, at least when temperatures are globally averaged and compared with the instrumental record for the past 125 years. The past century, as a whole, was also warm compared with the preceding 600 years starting just before the Little Ice Age. Moreover, the past several millennia are warm when held up against the previous 2.5 million years. Even so, most climatologists now predict that the warming that will occur by the middle of the next century will be greater than any experienced on Earth since before the development of the genus *Homo*. With that possibility in mind, a slightly more evolved group gathered at a recent convention* to discuss the characteristics and processes of past climates whose warmth matched or exceeded that anticipated for the near future.

Modellers and those who look at the geological record all provided a steady stream of evidence that, although carbon dioxide has almost certainly fluctuated throughout the Earth's history, the oceans have been important in regulating past global warming. Such news would come as little surprise to those who have been studying the role of the oceans in the glacial-interglacial cycles of the Pleistocene (the past 2 million years). But the importance of ocean transport in the Earth's warm periods has long been overshadowed by concern over the effect of CO₂. Now begins the formidable task of unravelling the exact role of the oceans and greenhouse gases, along with all their associated feedbacks, in the global warmth that dominated the past half-billion years.

Atmospheric carbon dioxide and ocean heat transport are the two primary candidates for regulating surface air temperature. Carbon dioxide arose as the new "paradigm" for past global warming (T. Crowley, Applied Research Corporation) when Barron and Washington¹ used a then state-of-the-art computer climate model to find if increased levels of CO₂ could yield a planetary warming of the magnitude indicated for the mid-Cretaceous (100 million years ago). Their experiments and more recent simulations of the Eocene (L. Sloan, Univ. California, Santa Cruz) show that, indeed, global warmth of the magnitude experienced throughout the past 200 million years can be achieved by increasing CO₂ to levels similar to those predicted by models of the global carbon cycle or estimated from geochemical proxies in the geological record. What the models also show, however, is that the latitudinal distribution of

surface air warming caused by increasing CO₂ is inconsistent with the patterns determined from palaeoclimatic data: CO₂ warms the Earth at all latitudes whereas the data consistently show warming to be greatly amplified towards the poles but marginal in the tropics (D. Poore, US Geological Survey, Reston; J. Zachos, Univ. California, Santa Cruz).

Intensified ocean circulation, taking heat from low to high latitudes, is viewed as the most likely mechanism for stabilizing the tropical temperatures under conditions of increased CO₂. Numerical experiments^{2,3} supported this, but indicated little further influence over global warming until a series of more detailed simulations showed otherwise. In particular, melting of the reflective ice caps as more heat is carried to the poles increases the amount of solar radiation absorbed by the globe. Indeed, it turns out that the increased ocean heat transport alone — that is, without any change in CO₂ — might have been responsible for the warm climates of the Mesozoic (245–65 million years ago) and Cenozoic (65 million years ago till now)⁴. Thus the question shifted from "what was the mechanism of past global warming?" to "which was the mechanism of past global warming?". Furthermore, climatological discussions, once built on the concept of the past as a key to the future, are now concerned with the validity of that concept.

Certainly there are many differences between ancient climates and the climate of the near future: the continents and oceans are distributed in completely different ways; continental topography has altered; the extent of ice over land is different; and vegetation patterns are not the same. Projects such as PRISM (Pliocene Research, Interpretations and Synoptic Mapping) are examining the most recent warm periods, seeking to minimize those differences in order to provide the best possible comparison between past and future (Poore). But PRISM's initial sea-surface temperature data for the North Atlantic⁵, and our model simulations using the PRISM data as boundary conditions (D. Rind and M.C.), now add more evidence suggestive of an oceanic source for the Pliocene warming, 3 million years ago. Furthermore, new sediment core carbon-isotope analyses (M. Raymo, Massachusetts Institute of Technology) reveal that middle-Pliocene CO₂ levels were at most 100 parts per million greater than they are at present (just over 300 p.p.m.). This is far below the 1,300 p.p.m. that our models at the Goddard Institute imply would be necessary to achieve

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the mid-Pliocene temperatures.

So if the oceans behaved so differently in past warm climates, is the past irrelevant to the predictions of future global warming driven by increasing trace gases? With different boundary conditions and different initial forcing mechanisms, different climates might be expected. There are several reasons not to be dismissive. Evidence of altered forcing by greenhouse gases in the Earth's history is ever more compelling. Ice-core records, carbon isotopes from palaeosols and marine microfauna, and carbon-cycle modelling all indicate that CO₂ change has influenced climate on geological time-scales. In addition, terrestrial data continue to accumulate showing that continental interiors, far from the oceans' influence, were substantially warmer, particularly during winter, in the Mesozoic and early Tertiary (K. Gregory, Lamont-Doherty Earth Observatory). Models have so far failed to reproduce this winter warming and increasing the amounts of greenhouse gases in the models is currently the only reasonable way to begin to reconcile this discrepancy (Sloan).

Although both the oceans and green-

house gases are behind the Earth's past warm periods, in a system as interspersed with feedback mechanisms as the climate, forcing factors may be far removed from resulting climatic states. The challenge now is not in determining cause and effect alone, but in understanding the series of processes connecting one transient state with the next. Global circulation models, working from the fundamental physical equations, may one day do the job, but not until atmospheric water and ocean features are simulated more realistically. Recognizing that the devil is in the details would ensure that the road to hell is not just paved with good conventions. □

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ENZYMES

Snapshots along the pathway

Mirosław Cygler

ON page 693 of this issue¹ a group led by Bauke Dijkstra describes a study of the reaction mechanism of the enzyme haloalkane dehalogenase through the crystallographic analysis of intermediates in the reaction. This is an ingenious piece of work, and highly informative for it provides snapshots of the enzyme in action.

Protein crystallography has provided a wealth of detail about the static structure of enzymes and has helped to delineate substrate binding sites, but it has told us little about the kinetic aspects of enzymatic reactions. Information about the intermediate states in a reaction is usually inferred from the structure of a stable complex of an enzyme with an appropriate model compound, a transition state analogue for instance. In the past few years technological developments such as intense X-ray synchrotron radiation sources and fast data acquisition techniques have provided the first glimpses of enzymatic reactions in progress. But Dijkstra and colleagues have now succeeded in monitoring an enzymatic reaction using conventional crystallographic methods. They have followed the breakdown of 1,2-dichloroethane by haloalkane dehalogenase through a series of stages: first free enzyme, then complexes with the substrate, with a covalently bound intermediate and with one of the breakdown

products remaining in the active site.

The difficulty in following transient processes with protein crystallography stems from the fact that the observed electron density distribution is an average over the time of the experiment and over all of the molecules in the crystal. The turnover time for a normal enzymatic reaction (well under 1 second) is much shorter than the data acquisition time. The alternative, investigation of stable enzyme-inhibitor complexes, does not provide all the answers as the study of the serine proteases shows — many complexes later, the details of the catalytic mechanism remain controversial^{2,3}.

Shortening data acquisition time from days to minutes and even seconds has become possible by combining synchrotron radiation with diffraction data collection by the polychromatic Laue method. Experiments by Hajdu *et al.*⁴ with glycogen phosphorylase *b* demonstrated that data collected in only a few seconds could provide an interpretable electron density map, showing clearly the binding of maltoheptose. But although in principle slow enzymatic reactions could then be visualized, synchronized action of all molecules in the crystal is required. This task has turned out to be rather difficult and, in current practice, only reactions with a half-life of the order of minutes are in-

vestigated. To that effect, Schlichting *et al.*⁵ used a photolabile derivative to unravel the details of the GTP hydrolysis by Ras p21 protein. The GTP derivative was co-crystallized with the protein and the release of GTP was triggered by flash photolysis.

The attempts to slow down reactions include the use of poor substrates, change of temperature or pH, and the use of caged substrates⁶. For instance, Sweet and colleagues⁷ applied synchrotron Laue photography to follow a deacylation step of a transient, trypsin-bound ester intermediate. By selecting a poor substrate and exploiting the pH dependence of the reaction to capture the intermediate and then release it slowly, they were able to identify the water molecule involved in the nucleophilic attack during deacylation.

An example of the application of a partially defective mutant to identify an intermediate state was provided by Strynadka *et al.*⁸. Following the observation that the Glu166→Gln mutation in RTEM-1 β -lactamase of *Escherichia coli* affects the release of covalently bound intermediate of β -lactam breakdown, they soaked the crystals of this mutant in penicillin G, collected high-resolution data using monochromatic synchrotron radiation, and characterized the intermediate state. From this structure and that of the free enzyme, they proposed a detailed reaction mechanism and identified the water molecules involved in the catalysis.

Dijkstra's group have now extended the use of standard crystallographic methods to follow the reaction catalysed by haloalkane dehydrogenase; this is one of many examples now emerging of a protein that has a fold in common with other proteins without having a significant similarity in sequence. The enzyme is one of the α/β hydrolase fold proteins, which are thought to have evolved from a common ancestor⁹. Their conserved catalytic nucleophile-histidine-acid triad, reminiscent of serine proteases, is adapted to hydrolyse vastly different substrates. Although the topological location of the triad is conserved, the nature of the nucleophile and the acid are not. Serine, cysteine or aspartate have been observed in the nucleophilic position, while both aspartate and glutamate have been found as the acidic member of the triad⁹. It is presumed that the ester hydrolysis by esterases of this family follows a mechanism similar to that delineated for serine proteases. But the possible enzymatic mechanism of dehalogenase, with an aspartate nucleophile, was less clear. Two possible mechanisms were consistent with the three-dimensional structure of the free enzyme. One involves a covalently bound intermediate, while the other is a general base catalysis by an activated water molecule.

By cleverly exploiting the effect of temperature and pH on enzymatic activity, the authors found conditions which allowed them to collect diffraction data from the enzyme during various stages of its catalytic cycle, using a conventional rotating anode X-ray source. By soaking the substrate 1,2-dichloroethane into the crystal for times ranging from hours to two days, and at various temperatures and pH, they were able to capture the substrate in the active site of the enzyme, an intermediate linked to nucleophile Asp124 through an ester bond, and a state where one of the products, a chlorine atom, is still present in the active site. All of these structures were determined at resolutions ranging from 2 Å to 2.4 Å, so the movement of solvent molecules could be followed.

After careful analysis of all these crystal structures, the authors present a detailed model of the reaction mechanism indicating the position of the nucleophilic water molecule, movement of the protons and the interactions with the protein that stabilize each of the reaction intermediates. Briefly, the 1,2-dichloroethane is first attacked at the C₁ atom by the O_{δ1} atom of Asp124. This methylene group is adjacent to the Cl-1 atom which is immobilized by the interactions with the NH groups of two tryptophan residues. The ester intermediate formed in this step is then attacked by a water molecule at the C_γ atom of the aspartate, forming a tetrahedral intermediate. The alcohol released in this step leaves first, whereas the Cl-1 atom freed in the first step of the reaction is the last to leave.

Altogether, this is a very impressive study — it shows how the manipulation of reaction conditions can be used to unravel mechanistic properties of enzymes by conventional crystallography. Combining this approach of slowing the reaction by conducting the experiment under unfavourable reaction conditions with another powerful technique, flash cooling to cryogenic temperatures, opens even more possibilities. One can foresee that these new ideas will enable us to watch other enzymes at work. □

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The rise and fall of *Rhizosolenia*

Thomas L. Hayward

PHYTOPLANKTON need to grow near the ocean surface, where sunlight drives photosynthesis. But the ocean nutrients they feed on are mostly a good hundred metres down, at the nutricline, and thus in the dark. On page 709 of this issue¹, Villareal *et al.* describe an algal self-service takeaway, or perhaps more accurately a marine dumb waiter, by which algal mats raise nutrients from the nutricline to where they can be consumed.

unexpected that a similar elevator could function in the open ocean. In any case, those dinoflagellates can swim; it is less obvious how *Rhizosolenia* controls its buoyancy, although Villareal and colleagues do have a speculative suggestion for how it could change — an ionic regulation mechanism like that found in *Pyrocystis*.

Apart from their changing buoyancy, a further hint that the mats shuttle between



Norbert Wu

FIG. 1 Weaving tangled webs — a mat of the diatom *Rhizosolenia*, roughly 3 cm across.

Their experiments involve observations of tangled mats of algae (the diatom *Rhizosolenia*; see photograph), often several centimetres across, collected by divers in the Pacific Ocean, and then suspended in sea water in glass jars. The mats, they found, rose or fell in the jars, and — more importantly — carried larger internal pools of nitrate on the upward part of their journey than on the downward part.

Why this interest in the lifestyle of algae? The processes governing primary production in the world's vast expanses of ocean must be unravelled to let us establish their role in the global carbon cycle. The disparity in the depths of the sunlit zone and the nutrient-rich regions has long been a puzzle. The obvious mechanism for nitrate transport would be diffusion from below, but the diffusive nutrient flux is only a small fraction of the required amount. This fresh proposal of Villareal *et al.* will be a welcome contribution to the debate.

These are not the first organisms suspected of vertical transport of nutrients — the dinoflagellates that make up massive, red-tinged 'blooms' in coastal waters have been suggested to sink at night to feed on nutrient-rich waters². But the distances involved are only a few metres. It is

the surface waters and the nutrient-rich depths comes from their nitrogen isotope contents. As Villareal and colleagues show, the mats have a high ratio of ¹⁵N to ¹⁴N, as does nitrate from below the nutricline, whereas the organic nitrogen being recycled in the upper waters contains more of the lighter isotope. It seems that the organic nitrogen being recycled at shallow depths is not, as has generally been presumed, the main sustenance for these algae; instead, they carry up nitrate from below.

As well as providing a fresh way of looking at an old problem, these results could help solve a new one. Recent observations of the particulate organic matter sinking from the euphotic (sunlit) zone show that it, too, seems to be enriched in ¹⁵N (ref. 3). This material was collected in sediment traps, and the first thought was that the traps might somehow preferentially collect organic matter that had been recently photosynthesized after an influx of nutricline waters. However, no nutrient inputs were observed, and it was unclear what sort of physical mechanism would mix nutricline water to shallow depths in the stratified open ocean⁴. The mechanism of Villareal *et al.* would close the ¹⁵N budget by providing an alternative shallow source of nitrate enriched in ¹⁵N.

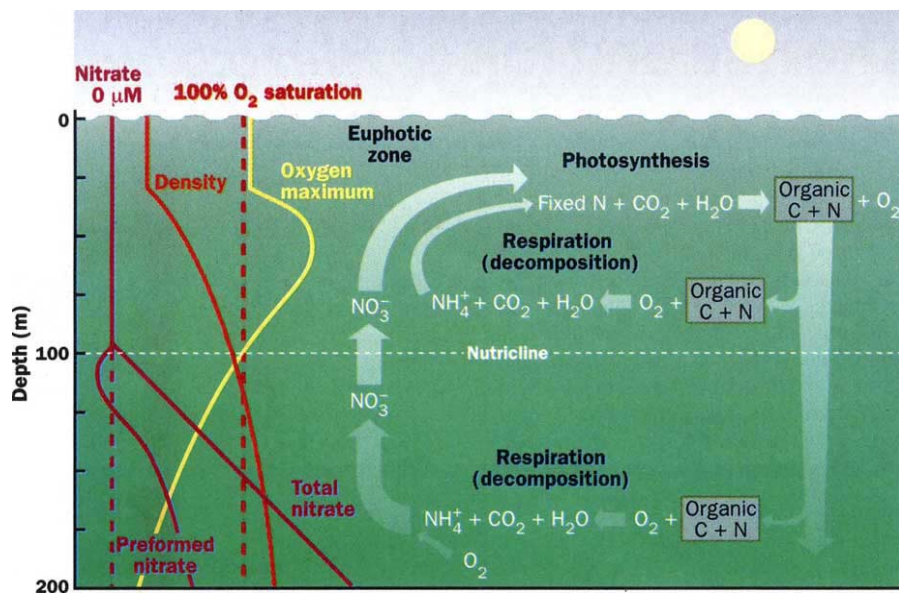


FIG. 2 Typical vertical profiles of density, total nitrate, 'preformed' nitrate and dissolved oxygen in the upper 200 m of the Pacific Ocean. *Rhizosolenia* mats², collected by divers in the upper 20 m, are found at depths much shallower than the nutricline (or sharp vertical gradient in nitrate). Vertical transport of nitrate to shallow depths by floating mats could provide the preformed nutrients required to form the shallow oxygen-maximum layer (the zone of supersaturated oxygen at about 50 m). The negative values of preformed nitrate at depths of 100–120 m (at the top of the nutricline) show that something is consuming it—either microbial activity, or the algal mats.

The new findings could also help to explain how a shallow layer of water supersaturated in oxygen is formed about 50 metres down. The gradual accumulation of oxygen implies that there is an extra input of nutrients over and above that taking part in the recycling system in the euphotic zone (photosynthesis using up nutrients and producing oxygen, decomposition and respiration consuming oxygen and generating ammonia). In the jargon, an input of 'preformed' nutrients is required. Such nutrients are lacking from the waters of the upper nutricline, so some nutrient source in addition to vertical mixing is required to create the supersaturated oxygen. My own recent hypothesis was that zooplankton migrating vertically transport nutrients contained in organic matter⁵. However, the vertical transport of NO_3 via floating diatom mats may be a less complicated alternative means of providing the required nutrients.

The process might explain yet another recent observation. Towards the top of the nutricline lies a layer that is surprisingly short of preformed NO_3 (see figure). This has been attributed to uptake of nitrate by microbes as they assimilate dissolved organic carbon⁶, but it could equally well be due to uptake of nitrate by migrating *Rhizosolenia* mats at this depth.

The debate is far from over. Some or all of the above processes may influence the transport and cycling of nutrients, oxygen and carbon in the upper ocean, but too few details are known for them to be

properly included in the basin-scale biogeochemical models. Can enough nitrate be transported up in this way, and is the 'elevator' fast enough? Calculation of the rate of NO_3 input from the mats is indirect and uncertain. It will depend on the abundance of mats, their migration rates and distance, and the excess NO_3 taken up per unit carbon. The distribution and vertical movements of *Rhizosolenia* mats are poorly known; they can certainly be collected at all depths reached by divers, but do they go deeper? They are rarely sampled in water bottles and, in the North Pacific, individual *Rhizosolenia* cells of the species found in the mats are normally found only at depths much shallower than the nutricline (E. Venrick, personal communication).

It may be that the broad investigation of the processes involved in nutrient cycling and carbon flux in the open ocean planned by the Joint Global Ocean Flux Study will answer some of these questions. Until then, the concept of the algal dumb waiter will provide much food for thought. □

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DAEDALUS

Military airlift

LAST week Daedalus proposed a novel sky-platform for communications and surveillance. It was based on the principle that the downwash of air from a helicopter spreads out beneath it in a conical fashion, and ultimately impacts a wide circle of land. A set of blowers distributed over such a circle, all aimed upwards towards the point of a cone, could create an exactly reversed upward flow of momentum. Converging to a point high in the sky, it could levitate a solid object at that point.

Daedalus is now working out how to get his platform up there. The obvious method is to start it at a very low altitude, and push it upwards by means of a narrow circle of extremely powerful blowers. As it rose, these would be turned down, while concentric circles of ever more distant blowers would be successively switched on. The whole sequence would be, in effect, the reversed-flow mirror image of the broadening and weakening of the high pressure area under a helicopter as it rises. The maximum height of lift would be determined by the radius of the largest circle of blowers. An array of 20 km across should be able to loft a platform about 10 km into the sky.

This simple airlift process cries out for other applications. At first, Daedalus hoped it could be used to launch spacecraft; but he soon realized that air-momentum transfer would fail as soon as the craft was rising much faster than the speed of sound. Even so, modern rockets burn about half their fuel to reach this speed, so airlift could replace much of the big expensive first stage. Asymmetric airlift could apply useful sideways thrust to an airborne platform. Accordingly, a simple passenger-carrying glider could be lifted to altitude and accelerated in any chosen direction, to glide down to an airfield hundreds of kilometres away. This could form the basis of a noiseless and terrestrially powered civil aviation system.

But Daedalus is drawn back to his original musings on air defence. A computer-controlled web of airlift blowers could be targeted devastatingly onto an invading aircraft. A concentrated blast of air-momentum could suddenly increase its lift or, in downward reverse-thrust mode, could cancel it. Imposed asymmetric flows of momentum could steer the intruder sideways out of its course, or even bring it to a dead stop in the sky, when it would fall like a stone. A widespread array of blowers would be hard to attack, and, anyway, could survive the loss of many elements. Apostles of non-violent resistance should approve.

David Jones

DNA from an extinct plant

SIR — The Dominican amber deposits have yielded a wealth of information about organisms living 25–40 million years ago in Hispaniola¹. We now report the extraction, amplification and sequencing of the chloroplast gene *rbcL* from a leaf of the extinct tree, *Hymenaea protera* Poinar (Fabaceae: Caesalpinioideae) (see ref. 2) in Dominican amber. Amber containing the leaf of *H. protera* (Fig. 1) originated from La Toca mine, between Santiago and Puerto Plata in the Cordillera Septentrional of the Dominican Republic.

Present knowledge about species diversity and distribution of the genus *Hymenaea* has been largely based on morphological characters. Lee and Langenheim³ divided the genus *Hymenaea* into a primitive *Trachylobium* section with *H. verrucosa* and *H. oblongifolia*, and a more advanced *Hymenaea* section with *H. courbaril*, concluding that an extinct ancestral stock gave rise to *H. verrucosa* and *H. oblongifolia*, with the latter giving rise to *H. courbaril*. We tested this hypothesis by using *rbcL* sequences from both extinct and extant taxa. We extracted DNA from leaf fragments of *H. protera* from the amber piece using previously described methods^{4,5} and from pieces of surface-sterilized leaves from herbarium specimens of *H. verrucosa*, *H. oblongifolia* and *H. courbaril* (supplied by J. Strother). Using primers complementary to conserved regions of the ribulose biphosphate decarboxylase *rbcL* gene, we amplified a 346-base-pair fragment flanked by the primers Z1 and Z346R, using polymerase chain reaction (PCR).

We sequenced PCR products by the dideoxy termination method and used all resulting sequences from *Hymenaea* spp. as queries in searches of the nucleic-acid sequences database using the MacVector 3.5 (IBI Kodak) DNA analysis program. We obtained high similarity

scores with all *rbcL* sequences present in the database, with the highest scores corresponding to those sequences from taxa in the family Fabaceae. Even higher scores were obtained by comparing the extant *Hymenaea* spp. with each other and with the extinct *H. protera*. Thus we conclude that the amplified product resulting from extracted *H. protera* was indeed from the *rbcL* gene.

We aligned sequences from *Hymenaea*

and *H. oblongifolia* than between *H. protera* and *H. courbaril*. This suggests that *H. courbaril* may be as old a taxon as members of the primitive section *Trachylobium*, but for some reason it has diversified much more rapidly than *H. oblongifolia*, which is restricted to the evergreen forest ecosystem of South America, and has remained fairly static over the same period.

This study essentially doubles the age of DNA that can be recovered and sequenced from extinct plants⁸. It shows that amber preserves the DNA not only

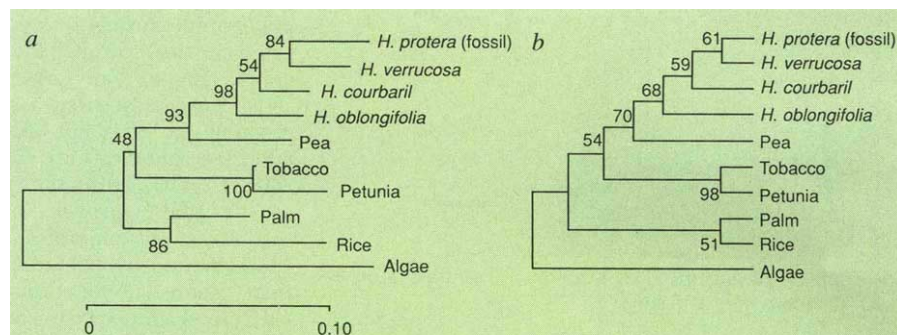


FIG. 2 Phylogenetic trees of extinct and extant taxa based on sequences of the *rbcL* gene, obtained by neighbour joining (a) and maximum parsimony (b). Numbers at the nodes of branches represent bootstrap confidence values obtained from 2,000 replications. Length of maximum parsimony tree (branch and bound method) is 176. Consistency index (CI)=0.807. CI, excluding uninformative sites, = 0.688. We corrected for transitions and transversions, resulting in identical tree topologies and nearly identical bootstrap confidence values. Sequences other than those from *Hymenaea* were extracted from the GenBank nucleic-acid database. The following taxa and accession numbers were used: Algae, used as the outgroup (*Chlorella*-like) Number M74441; palm (*Serenoa repens*) accession number M81815; petunia (*Petunia hybrida*) accession number X04976; tobacco (*Nicotinia glauca*) accession number M16867; rice (*Oryza sativa*) accession number X04789; and pea (*Pisum sativum*) accession number X03853.

spp. and other taxa to investigate phylogenetic analyses and to establish possible evolutionary relationships between extinct and extant *Hymenaea* spp. The aligned sequences were analysed by two methods, maximum parsimony (PAUP3; D. Swofford) and neighbour joining⁶ using the programs NJBOOT2 and Treeview (K. Tamura, Pennsylvania State University). Statistical confidence in the topologies was assessed using the bootstrap method, with 2,000 replications.

Our results corroborate earlier conclusions based on floral and vegetative morphology, indicating a sister-group relationship between the extinct Antillean species *H. protera* and the extant East African species, *H. verrucosa*⁷. This is supported by the high bootstrap confidence values (84%) obtained (Fig. 2), indicating a true phylogenetic relationship between these two species. At the same time, the data suggest that *H. protera* is more closely related to *H. courbaril* than to *H. oblongifolia* (based on distance calculations). Since *H. courbaril* is placed in the more advanced section of *Hymenaea*, we expected to see a closer relationship between *H. protera*

of invertebrates, but also the remains of angiosperms. Lavin and Luckow⁹ have recently concluded that there is a closer relationship between the flora of Africa and North and central America than there is between Africa and South America, which agrees with the conclusions from our new data on *Hymenaea*.

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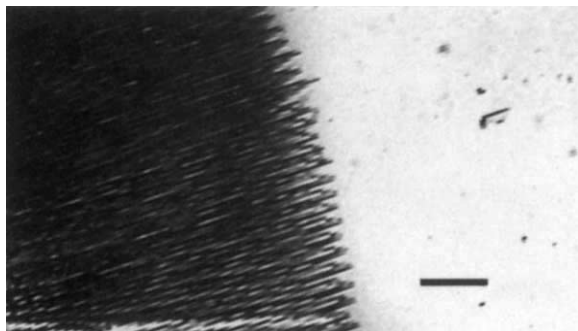


Photo by G. O. Poinar Jr

FIG. 1 Leaf of *H. protera* in amber from La Toca mine. The amber has been dated as 35–40 million years old.

Antifreeze protein in snake venom?

SIR — Rubinsky *et al.*¹ claim that the C-type lectin found in rattlesnake venom can inhibit ice growth in certain crystal planes and that the mechanism is probably similar to that of the antifreeze proteins in the body fluids of polar



Ice crystals growing in a 3% solution of xanthan gum at -10°C . Scale bar, 100 μm .

fishes. But the evidence is inconclusive. The authors were incorrect in their belief that modification of the growth habit of ice crystals by binding to the crystal surface is unique to fish antifreeze proteins. Non-colligative antifreeze proteins have been identified in several invertebrates² and antifreeze proteins have also been found in the leaves of cold-acclimated winter rye (*Secale cereale* L.)³.

In the natural world, antifreeze proteins have two functions: to depress the freezing point of fluids to below the surrounding temperature to prevent ice formation; and to suppress ice recrystallization once ice has formed⁴. The two methods generally used to test for the presence of antifreeze proteins are based on these two properties. Thus antifreeze proteins are either identified by measuring their non-colligative freezing point depression⁵ or by measuring rates of recrystallization⁶. Rubinsky *et al.*¹ used the first method, the determination of freezing point using a Clifton nanolitre osmometer, but did not detect any non-colligative freezing point depression. They did not use the second method.

To support the view that C-type lectin acts like an antifreeze protein, Rubinsky

et al. showed that the ice crystals grown in lectin solutions are spicular and resemble those grown in a sea raven antifreeze protein solution. But ice crystals of this shape are quite common in many solutions grown in various circumstances.

Muhr and Blanshard⁷ have shown photographs of clouds of ice crystals in the form of straight dendrites (very fine parallel needles) which were grown in a solution of sucrose, alginate and calcium hydrogen phosphate. Tiller⁸ grew ice crystals in a solution of potassium chromate and produced fine parallel filaments. Rapatz and Luyet⁹ show parallel ice 'spears' growing in partially dehydrated muscle fibre at -16°C . The figure shows ice crystals grown at -10°C in my laboratory. The solution is 3% xanthan gum, a commonly used food thickener. The

crystal shapes are almost identical to those shown by Rubinsky *et al.* Thus the shape of ice crystals depends on various physical and chemical factors and is not a reliable indication of non-colligative freezing behaviour.

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Phytoplankton growth and CO_2

SIR — By making a case for CO_2 limitation of phytoplankton growth in the sea, Riebesell *et al.*¹ focus long overdue attention on the role of CO_2 in marine phytoplankton ecology. Their case, however, is equivocal. Their theoretical analysis assumes that diatoms use only CO_2 and that the production of CO_2 from HCO_3^- is uncatalysed. These assumptions may be valid for some species but require rigorous testing in the light of work showing that *Phaeodactylum tricornutum* produces extracellular carbonic anhydrase² and, together with some freshwater diatoms, has the capacity to utilise HCO_3^- (refs 3–5). Although CO_2 may be a growth-rate-limiting resource, the experimental design of Riebesell *et al.* altered CO_2 concentrations by varying pH. Therefore, it is unclear whether the observed changes in growth rates are caused directly by changing pH or indirectly by CO_2 .

A more general point relevant to future attempts to interpret stable carbon isotope ratios in phytoplankton is the

need to distinguish between CO_2 -limited photosynthesis and CO_2 -limited growth. CO_2 -limited photosynthesis does not necessarily imply CO_2 -limited growth provided that: P_{max}/μ is greater than or equal to the reciprocal of the fractional limitation of photosynthesis by CO_2 availability, where P_{max} is the CO_2 -saturated rate of carbon-specific net photosynthetic carbon fixation; and μ is the carbon-specific growth rate.

In cyanobacteria and green algae, HCO_3^- and/or CO_2 transport are induced in response to low levels of dissolved inorganic carbon. Full induction may occur without any appreciable decline in steady-state growth rate^{6,7} even though such cells exhibit steady-state CO_2 -limited photosynthesis. A decrease in discrimination against $^{13}\text{CO}_2$ corresponding to the induction of the CO_2 -concentrating mechanism has been observed⁸. Consequently, CO_2 -limited photosynthesis can explain the striking inverse relationship between stable carbon isotope composition of marine phytoplankton and ambient CO_2 concentrations^{9,10}. But, in isolation, this does not imply that CO_2 limits phytoplankton growth rates in the sea.

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RIEBESSELL *ET AL.* REPLY — Turpin makes two important points pertinent to the question of CO_2 limitation of phytoplankton growth. With respect to his first comment, we agree that inorganic carbon use by the common marine diatom species needs to be rigorously tested. With the exception of *Phaeodactylum tricornutum*, a brackish water species which in many aspects is an atypical diatom, information on carbon acquisition of marine diatoms is not available¹¹. We have now analysed sixteen species of marine diatoms and two species of Prymnesiophytes for their ability to use bicarbonate (manuscript in preparation). We found no evidence of HCO_3^- use for any of the planktonic diatoms or the Prymnesiophytes. Their rates of carbon uptake were consistently lower than the maximum potential supply of CO_2 over a wide range of CO_2 concentrations. Both HCO_3^- use and the presence of extracellular carbonic anhydrase would result in uptake rates higher than the rate of uncatalysed CO_2 supply at low ambient CO_2 concentrations. We did find evidence of HCO_3^- use for one benthic diatom species. These results further substantiate the assumption of uncatalysed CO_2 use of marine planktonic diatoms.

Based on the information presently available, the question of whether the observed change in growth rate of the

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three diatom species we tested¹ occurred merely in response to alterations in the CO₂ concentration, or whether it was also influenced by the concomitant change in pH, cannot be resolved. However, analogous to our experimental design, under natural conditions any change in the CO₂ concentration of sea water inevitably goes hand in hand with a change in pH. Thus, a distinction between a CO₂- and a pH-related response in growth rate is not critical for extrapolation of the experimental results to natural conditions. Naturally, we agree that from a physiological point of view this question is of particular interest.

The second point raised by Turpin, the need to distinguish between CO₂ limitation of photosynthetic rate and growth rate, is indeed relevant to the interpretation of phytoplankton stable carbon isotope compositions. Because we actually measured growth rate rather than short-term photosynthetic rate, however, our results provide neither a means nor a need to distinguish between

the two. In fact, the observed CO₂ limitation of growth rate¹ also implies CO₂ limitation of photosynthetic rate.

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ing that Glatt and Snyder¹¹ did not detect type 5 mRNA in the heart. Our solution hybridization data are in agreement with those of Ishikawa *et al.*⁵, which indicate that type 5 adenylyl cyclase mRNA is quite abundant in the heart.

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Type 5 adenylyl cyclase distribution

SIR — Several groups have reported the identification of eight G_s-sensitive adenylyl cyclases^{1–11} which can be classified into five distinct families. One group reported that an adenylyl cyclase cloned from a striatal complementary DNA library is brain-specific and localized to the striatum¹¹. We⁶ and others⁵ had cloned the same adenylyl cyclase and called it type 5. The tissue specificity of the expression of one type of a widespread G

protein effector, such as adenylyl cyclase, is of potential importance.

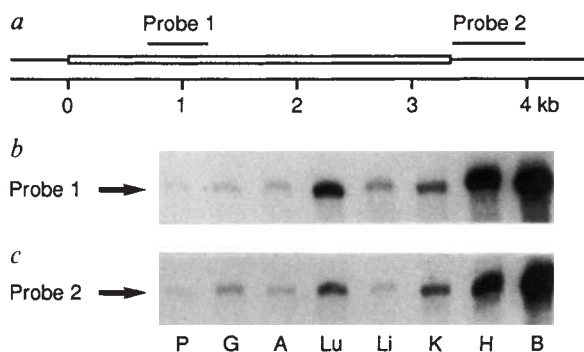
Previous studies using PCR (polymerase chain reaction) had indicated a wide distribution for the type 5 and the related type 6 adenylyl cyclases^{6,9}. It could be argued, however, that PCR is too sensitive and would detect very small and biologically inconsequential amounts of mRNA. Thus, we used solution hybridization to determine the presence of type 5

adenylyl cyclase mRNA in various tissues (see figure). Our probe 1 is a 500 base-pair fragment within the coding region, where the nucleotide sequence of the type 5 adenylyl cyclase is 82% similar to type 6. Probe 2 (a 620 base-pair fragment) encodes the 3' untranslated region¹² and is unique for the rat type 5 adenylyl cyclase (a in the figure). Total RNA from various rat tissues was extracted, and 10 µg from each tissue was used in the solution hybridization experiments. Both probes yield very similar patterns (b, c in the figure). We find that messenger RNA encoding type 5 adenylyl cyclase is most abundant in brain and heart, and is also found in pancreas, gut, adrenal gland, lung, liver and kidney. It is therefore surpris-

ing that Glatt and Snyder¹¹ did not detect type 5 mRNA in the heart. Our solution hybridization data are in agreement with those of Ishikawa *et al.*⁵, which indicate that type 5 adenylyl cyclase mRNA is quite abundant in the heart.

In the pituitary gland we observe the enzyme localized particularly to the anterior lobe. In the kidney, messenger RNA encoding the enzyme is higher in the medulla than the cortex, and associated with tubules. In the eye, the striatal adenylyl cyclase is localized to the retina, both results agreeing with our initial conclusion that the striatal adenylyl cyclase is associated with dopamine's actions¹. Thus, dopamine neurons and receptors are localized in the retina of the eye⁴, in the atria and great vessels of the heart⁵, in the anterior pituitary gland⁶ and in the kidney⁷.

Pleroni *et al.* have conducted solution hybridization techniques and detect RNA



Detection by solution hybridization of mRNA encoding type 5 adenylyl cyclase in various tissues of the rat. a, Location of probes along the cDNA for type 5 adenylyl cyclase. b, Solution hybridization analysis of total RNA from pancreas (P), gut (G), adrenal gland (A), lung (Lu), liver (Li), kidney (K), heart (H) and brain (B) using probe 1. Type 5 enzyme mRNA is detected in all tissues. c, Solution hybridization analysis using probe 2. RNA was isolated by the guanidinium thiocyanate method. ³²P-labelled cRNA probes were prepared, and their sizes were verified by electrophoresis on an acrylamide gel. Samples of total RNA (10 µg) from each tissue were analysed for the presence of type 5 adenylyl cyclase mRNA by solution hybridization/RNase protection assay. After digestion, the samples were resolved on an acrylamide gel. Autoradiographs of the gel are shown.

for the type 5 adenylyl cyclase most highly concentrated in the brain, but with readily detectable levels in the heart, kidney and lung, raising the question as to why our northern blot analysis did not show the cyclase in these peripheral tissues. They used whole rat brain, of which the corpus striatum is only about 5%. In their analysis levels in whole brain are roughly 5–10 times those in the peripheral tissues. Thus, striatal levels of the enzyme are about two orders of magnitude higher than these peripheral tissues. At the exposure times we used we would not have detected a signal in tissues that was only one-hundredth that of the corpus striatum.

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Health risks and natural gas

SIR — We have found that oxide coatings on gas burners in Polish houses 5 to 100 km away from gas deposits in the Rotliegendes basin contain high concentrations of Pb, Cu, Ag and As. This long-distance transport of metals takes place presumably in the form of stable gaseous organometallic compounds. The Hg, Pb and As content of natural gas from the Rotliegendes basin poses a health hazard with respect to its domestic use.

Natural gas in Permian and Carboniferous rocks in the central part of the Proterozoic basin in Poland contains Hg in concentrations equal to the saturation limit¹. Underground sections of pipes used for gas extraction are often heavily corroded and coated with native lead, lead amalgams, native copper and its amalgams with Ag and Fe–As alloys.

Oxide coatings on gas burners were collected from kitchens in Poznan, Buk and Siekierki (Poland), and one reference coating in Vienna (see table). The Vienna location was probably supplied by a field in Siberia, 3,000 km away. Oxide coatings in Poland were abraded from the surface with a file, and the oxide coating on the gas burner from Vienna was dissolved by submerging the gas burner head in aqua regia. The

CONCENTRATION OF METALS IN OXIDE COATINGS OF GAS BURNERS IN KITCHENS

Locality (town)	Pb(p.p.m.)	Ag(p.p.b.)	Cu(p.p.m.)	As(p.p.m.)	Distance to deposit (km)
Siekierki	112	98	34	15	20
Buk	491	1,082	57	32	5
Buk	511	1,321	61	45	5
Poznan	134	82	21	≤5	~ 100
Vienna	1,283	1,136	61	?	3,000?

Determined by atomic absorption spectroscopy.

second technique gives higher metal contents than the first, probably because filing of gas burner coatings significantly contaminates the sample with underlying iron, which lowers the measured concentrations of the other metals owing to dilution.

We found that the concentrations of Pb, Cu, As and Ag in all the oxide coatings from Poland are very high (see table). The metal contents are roughly a function of the distance to the gas source, being highest in Buk. The metal contents in oxide coatings in Vienna are also very high (see table). This might be due to the different technique used to collect the Vienna specimen, although it is also pertinent that the gas burner in Vienna has been in use for about 25 years while those in Poland have been in use for only about 10 years.

The transport of heavy metals over long distances could possibly be due to the formation of volatile organometallic complexes. The most suitable compound to transport lead in methane is tetramethyl lead ((CH₃)₄Pb). Gas chromatograms of metal precipitates found in underground sections of gas pipes indicate the presence of higher hydrocarbons, but also some traces of hydrocarbons with a very high molecular weight were released at temperatures of 100–140 °C. More precise identification of the nature of these hydrocarbons trapped in the metal precipitates requires further study.

Methyl compounds of Ag and Cu may be stable in the presence of tetramethyl lead. This may explain the relationship between Pb and Ag, and Cu concentrations in gas burner coatings. The methyl compound of arsenic ((CH₃)₃As) melts at –130.5 °C and boils at 50 °C, but is unstable². This instability may be reflected in the low As content of oxide coatings on gas burners. Some mercury has also been detected in the oxide coatings, but the elemental matrix caused difficulties in obtaining low limits of detection and analytical repeatability. Methyl mercury ((CH₃)₂Hg), a very stable compound, boils at 92 °C and is also very poisonous². Therefore mercury could be transported long distances not only as mercury vapour, but also as gaseous (CH₃)₂Hg. Further studies are clearly needed to validate these hypotheses.

Methyl complexes of Pb and Hg are

much more poisonous than the metals. Without demercuration of the Rotliegendes basin gas, the mercury content in the air in kitchens during cooking may exceed 23–26 times the safety limit¹. No measurements have yet been done of lead content of gas or of air in kitchens. The data presented in the table are preliminary, but we believe that the high toxicity of the methyl derivatives of Pb and Hg calls for an urgent further study.

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Pointing the way

SIR — Recent reports of the presence of interesting words in protein-sequence databases^{1–3} have encouraged me to mention a further example concerning the formation of disulphide bridges in the S-rich prolamin storage proteins. Using a similar algorithm to that reported by Harvey³, using a human neural network, we have discovered the suggestive sequence SSLINKASE, which forms the carboxy-terminal amino acid sequence of an oat prolamin⁴. To emphasize the message, the seven letters immediately preceding this sequence — PEGEDE — form the past tense of the Danish verb 'to point'.

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Jaws with claws

Henry Gee

Jurassic Park. A film directed by Steven Spielberg. Written by Michael Crichton and David Koepp. Released by Universal: 1993. Opens in Europe in July.

LIKE all good science fiction, Steven Spielberg's latest film epic *Jurassic Park* takes established science and pushes it just a little further than reality currently allows. It helps that the science is a potent mixture of dinosaurs (dim-wittedly omitted by Hilaire Belloc from *A Bad Child's Book of Beasts*), genetic engineering (aired with mingled fascination and disapproval at the dinner tables of trendy would-be theologians everywhere) and chaos theory (purveyor of psychedelic fractal images to the grunge generation).

The story goes like this. John Hammond (Richard Attenborough) is an eccentric Barnum-like millionaire who rents an island off the coast of Costa Rica with a view to making it a Mesozoic theme park — with real dinosaurs, cloned using the most sophisticated equipment money can buy ("no expense was spared" is his constant reminder). Armed with the kind of supercomputing and gene-sequencing hardware rarely seen outside a national laboratory, Hammond's scientists extract dinosaur DNA from the gut contents of mosquitoes interred in Mesozoic amber, amplify it, sequence it and stitch it together, the large holes filled with the appropriate nucleotide sequences from frog DNA. The reconstituted genomes are folded into doctored crocodile ova and left to mature within special plastic eggshells. With a little care and attention, the end result is a dinosaur squab.

The story begins with the park fully stocked with dinosaurs but not yet ready to be opened to the public. For a weekend of frank admiration, Hammond flies in a few of the scientists whose work — bank-rolled by Hammond — has without their knowledge made the whole thing possible. A lawyer who needs to be satisfied of the park's safety procedures comes too (there have already been gory deaths among the workforce).

Thus arrives a gaggle of realistically awkward scientists — shy palaeontologists Alan Grant (Sam Neill) and Ellie Sattler (Laura Dern) set off by Jeff Goldblum's brash chaos theorist Ian Malcolm, who behaves like a rock star (he looks, in fact, like an Israeli entry for the Eurovision song contest). Oddly, Malcolm's function is to play Cassandra, warning that, through the butterfly effect, the elaborate theme park is destined to go horribly wrong, which it does, of course. No Spielberg film is complete without a pair of irritating children, and this is no exception — Hammond's grandchildren arrive to

complete the house party.

The scientific scheme is not completely outrageous; unless one looks too closely, there is nothing impossible about any of it in theory. Hammond takes his guests on the tour that would greet genuine visitors, during which they are privileged to witness the best popular explanation of DNA and cloning I have ever seen, in the form

though stylish exit replaces the egregious gore of Michael Crichton's novel, on which the film is loosely based. To be eaten alive by a *Tyrannosaurus* is unfortunate: to be so eaten while you are on the lavatory is undignified as well.

This is just the kind of superior all-action adventure that Spielberg perfected long ago in his *Indiana Jones* films, but with the addition of dinosaurs. The science, though treated extremely well, is only stuffing to ease the suspension of disbelief. After a ragged and hesitant first half-hour, the film jams into full throttle and roller-coasters all the way to the very end. Monsters jump from behind every bush and tree with toothy menace. Although they are dinosaurs (and con-

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IMAGE
UNAVAILABLE
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REASONS

From eggs-tinct to eggs-tant — a dinosaur hatches out in *Jurassic Park*.

of an animation starring that soon-to-be-famous character 'Mister DNA' — a good way to get round the leaden monologue that usually passes for scientific explanation in films (museum exhibition designers should take note). In a clever and witty script, the characters pose the questions we ourselves would like to ask ("What about mitotic arrest?" asks Grant), only to have them gently pooh-poohed, reinforcing the impression that, given enough money and hardware, even the most difficult problems can be solved with ease. But only the lawyer is really impressed by the flashing lights. The scientists, although initially bowled over by the sight of real live dinosaurs, start asking difficult questions about hubris.

Then things start to go awry. To cut a long story short, treachery in the computer department leaves the electric fences switched off, and the guests marooned in the park at the mercy of big fierce *Tyrannosaurus*, small fiercer *Velociraptor* and their dentally advantaged friends. The rest is predictable. The bad guys get despatched in nasty ways, al-

vincing, too), they could have been any death-dealing automata for the film to have been a good example of its genre: substitute hostile extraterrestrials, lunatic Nazis or predatory androids and it would have been the same film with a different title — *Aliens*, *Raiders of the Lost Ark* or *Terminator 2*.

Nevertheless, Spielberg's magic takes it a hair above the competition. The direction is superb, the script taut and bursting with *Indiana Jones*-like one-liners and (thankfully) irony and humour take the place of schmalz, sex and rude words. It is, though, incredibly violent.

Critics will carp at the predictability of it all, the gaping holes in the plot, the occasional woodenness. Ignore them. Despite rapid temporization in the cause of credibility among their peers when the preview ended, even the most dyspeptic hack was ooh-ing and aah-ing along with the rest of us while the film was actually playing. Go and see it. It's terrifying. And wonderful. □

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Users and producers

Philip Gummett

The Second Culture: British Science in Crisis — The Scientists Speak Out. By Clive Cavendish Rassam. *Aurum*: 1993. Pp. 242. £16.95.

WHILE claiming the idea for this book as his own, the author thanks Professor Denis Noble and Dr John Mulvey for suggesting lines of enquiry and "correcting one or two mistaken impressions that I may have had". It is appropriate to mention this acknowledgement because, although it does not so claim, the book could be read as a contribution to the Save British Science campaign which has been so ably led by Noble and Mulvey. It is a cry from the heart about the state of British science, supported by copious quotations from leading scientists. Whether it will help is a more open question.

Rassam's point of departure is C. P. Snow's Rede lectures in 1959 on the "Two Cultures". Hence, he follows a line of argument that attributes the alleged undervaluing of science in Britain, and the malaise in the British economy, to the lowly esteem in which scientists are said to be held. He then acts as lawyer for the prosecution, seeking supporting evidence primarily from interviews with more than 70 scientists from a wide range of specialisms. Among their number are heads of universities or Oxbridge colleges, senior industrialists, the head of a research council, Nobel laureates and fellows of the Royal Society. This leads him into the most novel part of the book, in which he draws pen pictures of "lives in science", ranging across seven disciplinary areas. His fascination with his subjects, and how they made their crucial breakthroughs (often in ways that run against naive arguments about planning science), is evident, as is his sympathy with their tales of woe about today's funding of science.

The pen pictures might be fine as individual journalistic pieces. Some contain fascinating nuggets. But it is not clear whom the author expects to read diligently through dozens of them. Indeed, what is the target readership? A main aim of the book, we are told, is to make science and scientists much less remote. But those wanting to persuade a nonscientist of the adventure and uncertainty of science could find more accessible material by, say, Peter Medawar, James Watson or Arthur Koestler.

Perhaps, then, the real aim is to persuade policymakers of the need for change? In that case, I fear that the book would not pass muster in Whitehall. This is not because of any anti-science bias, but

because the case has to be argued better than it is here.

A hypothetical Whitehall reader might first remark on a failure to assess critically the evidence presented. We are told that one in three Britons believes that the Sun moves around the Earth without being told that in the United States the position is at least as bad. We are also told that whereas American economists have at least tried to understand the determinants of technological change, there is "scarcely a book by a British economist which mentions science in a significant way". This will surprise those British economists who are widely regarded as world leaders in this field. It is also indicative of a general lack of awareness in the book of the substantial British and international literature on science and technology policy that could have added substance to many of the arguments.

There are many factual errors. To mention a few: Sir Ieuan Maddock was not "the government's chief scientist"; the Centre for the Exploitation of Science and Technology was not set up as a result of the Alvey initiative (there is no mention of the Advisory Council for Applied Research and Development report that was more directly instrumental); and it was not "during the 1970s that the costs of science began to grow significantly faster than inflation" — the Advisory Council on Scientific Policy (which did not comprise largely representatives of the research councils) had identified this problem more than a decade earlier. Nor do such remarks as "it was therefore to be expected that many scientists would vote Conservative in 1979" or "it is said that in the Leeds

university chemistry department the average age is 58" necessarily convey a sense of solid research. The logic of a position that on one page presents it as a "problem" that academics seeking funding under the Alvey programme had to have an industrial partner, and on the next page cites it as a benefit of the programme that it stimulated university-industry collaboration, is also puzzling.

Rassam's principal recommendation is that a statutory Science Directorate should be set up outside politics (like, he suggests, the British Broadcasting Corporation and the Arts Council), with the power to fund on a 3–5-year timescale the research councils, universities and a range of strategic and applied research areas. He does not show how this would deal with what I take him to regard as one of the key economic issues: namely, the stimulation of industrial demand for the research at which, as he shows, Britain is still so adept. In any case, with the publication in May of the White Paper on the future of British science and technology policy, the debate has moved on. But the issues remain, and in what can only be regarded as a long haul to strengthen the research council system through the new arrangements in the Office of Science and Technology, and to seek fruitful harmonization of users and producers of research through the proposed exercises in technology foresight, the themes addressed in this book will continue to resonate. □

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Taking different sides

Michael C. Corballis

Hemispheric Asymmetry: What's Right and What's Left. By Joseph B. Hellige. *Harvard University Press*: 1993. Pp. 396. \$41.95, £27.95.

THERE are two kinds of book about the two sides of the brain. One kind, a product perhaps of the right brain itself, promotes the now familiar view that the right brain is artistic, intuitive and creative, while the dull old left brain is logical, rational and symbol-bound. These days, this message is more likely to be directed at art teachers or marketing executives than at experimental psychologists or neuroscientists. *Hemispheric Asymmetry* represents the other kind, a careful marshalling of the evidence, intended "to sort the facts from the fantasy of hemispheric specialization". Its stance is deliberately ahistorical; what Joseph Hellige hopes to achieve is "a freeze-frame view of our current

state of understanding".

There may, however, be perils in ignoring history. Interest in cerebral asymmetry was first awakened in the 1860s when Broca reported evidence for the left-cerebral dominance for speech, and a flurry of research and speculation ensued. Fantasy quickly overwhelmed fact, and the topic fell largely into oblivion around the turn of the century. In the 1960s, history repeated itself when R. W. Sperry carried out his Nobel-prizewinning studies of the so-called 'split-brain' patients, leading to the left brain/right brain cult that is now ingrained in our folklore. Part of Hellige's mission is to "anticipate what the next frame might look like", but with another century about to turn, history warns us that the next frame might well be empty.

The book is nevertheless a clearly written, modern account of functional and anatomical asymmetries in humans and

nonhuman animals. It is not likely to appeal to executives seeking new marketing slogans or opportunists of the New Age trying to convince a gullible public of miraculous powers in the right brain, but it will inform those who want to know what is really going on in the field. A recurring theme is that human thought is organized into subsystems, or modules, that depend on different brain structures, and this greatly complicates the search for simple principles. For example, language is generally regarded as the epitome of left-brain function, but detailed analysis shows that the right brain also contributes in important ways, especially to the pragmatic aspects of language.

Although much of the evidence has come from brain-injured patients, the book dwells more on techniques to assess cerebral symmetry in intact individuals. One main technique exploits the fact that each side of the visual world projects to the opposite side of the brain; indeed, over the past 30 years or so, experimental psychologists have established a virtual cottage industry in comparing processing efficiency between the two visual half-fields. But while this may seem to provide a convenient mirror to our asymmetrical brains, the picture it reflects is far from clear. No single principle seems to capture the increasingly intricate findings, and Hellige again resorts to multiple modules, a theoretical strategy that sometimes verges on tautology. Even so, the author is a leading researcher in this area, and guides the reader patiently through the maze of theories and experimental frameworks. His account will give budding students a good feel for the ways in which questions are formulated and addressed experimentally, but those seeking immediate answers may well experience a sense of frustration. Fact may not have yielded to fantasy, but it has nevertheless proved peculiarly elusive.

Other topics, such as anatomical and biological asymmetries in the brain, asymmetries in nonhuman species and individual variations in asymmetries, are given more superficial treatment, but are as a consequence more readable. There is also a chapter on evolution, now seemingly an obligatory topic in discussions of cerebral asymmetry. Hellige's version relies a little too much on secondary sources, but is nevertheless nicely balanced. This more expansive view of the role and nature of hemispheric asymmetry is in marked contrast to the narrowing focus of the chapters based on the author's own research interests, but it is the broader view that may well save hemispheric asymmetry from oblivion when the century turns. □

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Bursting terrestrial bonds

Stuart Ross Taylor

To a Rocky Moon: A Geologist's History of Lunar Exploration. By Don E. Wilhelms. University of Arizona Press: 1993. Pp. 477. \$29.95, £26.99.

DON Wilhelms is best known as the author of the definitive *Geologic History of the Moon*, published by the US Geological Survey (USGS) in 1987 (Professional Paper 1348). Now he has written a personal account of the history of lunar exploration in which he recounts the background to how we came to understand the geology of the Moon. It is a fascinating tale of human endeavour, re-

of duplication. Much turned on the personalities involved, such as Gene Shoemaker who operated the successful Astrogeology Branch of the USGS at Flagstaff, Arizona, and Dale Jackson who unsuccessfully attempted to plant the USGS flag at NASA's Manned Spacecraft Center (now the Johnson Space Center) at Houston, Texas. The book is a detailed and accurate source of information on this, and much else, for students of the history of the Apollo project, and provides a unique insight into how the whole affair was conducted. The author's description of the personalities is frank, warts and all. Eventually

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Late nineteenth-century German engraving of the lunar surface.

sourcefulness, error and the emergence of the truth.

It's all there: Harold Urey (surely one of the heroes) and his quarrels with the geologists, and finally his acceptance that the huge plains — or maria — visible as dark markings on the surface of the Moon are covered with basaltic lavas; the rear-guard action fought by those who saw lunar craters as volcanic; and Tommy Gold's insistence on dust-filled maria, into which the spacecraft would sink. Transient phenomena, the observations of apparent current activity on the Moon observed through telescopes but not by the astronauts, receive scant treatment: "whatever the real or psychological cause of transient phenomena, it is not volcanism". It is sobering to recall how little was agreed on before we went to the Moon.

Relations between the USGS, who maintained that the study of rocks, from whatever source, was its business, and NASA (National Aeronautics and Space Administration), which felt a proprietary right to lunar samples, were generally strained. Informal agreements led to a lot

of the relationships all came good. The combination of patient mapping and the study of returned samples enabled the geological history of the Moon to become so well established that we now understand it better than that of the Earth.

Wilhelms begins his account of lunar studies in August 1892, when G. K. Gilbert, then chief geologist of the USGS, spent an informative 18 nights observing the Moon through a 67-cm telescope. This was the beginning of the correct geological interpretation of the lunar surface, and was followed by Ralph Baldwin's *Face of the Moon* in 1949; but there were many false leads, both in between and later, principally from workers who considered that the great craters were volcanic. It took plenty of work before it was accepted that the lunar plains or maria were indeed basaltic lavas, but that the basins and craters that they filled had been excavated much earlier by meteorites or asteroids, as Baldwin had thought.

Nevertheless, the geologists were not always correct; doing geology from photographs has its hazards. The most notorious

episode was the pre-mission identification of the hilly Apollo 16 landing site at Descartes as a collection of volcanic landforms; it turned out to lie in the middle of the heavily cratered lunar highlands. "The moral of the story of Apollo 16 is that investigators should have stood back and viewed the big picture. . . . Basically, we goofed. . . . Geologists were too captivated by terrestrial analogues", says the ever-honest and dispassionate Wilhelms. This theme, that comparisons with the geology of the Earth usually prove misleading, occurs throughout the book. Wilhelms's judgement that the Moon is not a little Earth should be heeded by all those engaged in the currently fashionable study of comparative planetology.

The Apollo missions are treated in detail, with much understanding of the difficulties of fieldwork by the astronauts in an alien terrain. Some missions were better than others; the Apollo 15 flight to Hadley Rille at the foot of the Apennine Mountains was perhaps the most outstanding. Wilhelms laments, as we all do, the political decision to cancel the three final missions (Apollos 18–20, for which the hardware existed) to such mouth-watering sites as Copernicus, Gassendi and the Marius Hills. Even the spectacular rayed crater Tycho, deep in the southern highlands, would have been accessible at that stage to a manned landing. In the event, the final mission to return samples was the Soviet Luna 24 unmanned flight to Mare Crisium in 1976.

The successes of the Apollo missions have caused us to forget the many hazards involved. In unemotional prose, Wilhelms provides the best account I have read of the drama of the first lunar landing. As the Apollo 11 astronauts were on their final descent to the surface of Mare Tranquillitatis, the onboard computers signalled faults with the lunar lander. The cool-headed navigation controller at Houston, a 26-year-old MIT graduate, correctly judged the alarm signals to be due to computer overload, and the landing went ahead. Wilhelms notes that the Apollo missions, with all their complex technology, suffered many fewer problems than the later Space Shuttle programme.

He also answers the inevitable question 'Could less costly automated return of samples have accomplished the same results as the manned missions?' with a frank no. This view coincides with the considered opinion of most scientists. After all, documented rock samples, and not merely soil and small rock fragments as returned by the Soviet Luna unmanned missions, were needed to obtain correct isotopic ages and to unravel the history of the Moon. □

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The outsider

W. F. Bynum

Archibald Garrod and the Individuality of Man. By Alexander G. Bearn. *Oxford University Press: 1993. Pp. 227. £35, \$65.*

To many, Archibald Garrod (1857–1936) is at best a name associated with a phrase: 'Inborn errors of metabolism', the title of his 1908 Croonian lectures to the Royal College of Physicians, subsequently published as a moderately sized volume. Even in his own lifetime, Garrod never achieved the visibility that the outward trappings of a highly successful career might have bestowed: consultancies at the



Garrod — metabolic sleuth.

Hospital for Sick Children in Great Ormond Street and at St Bartholomew's Hospital in London; co-authorship of a pioneering textbook of paediatrics; fellowship and the vice-presidency of the Royal Society; a knighthood; directorship of the first academic medical unit to be established in a British medical school, at St Bartholomew's; and the Regius Chair in Medicine at Oxford, in succession to his friend Sir William Osler.

Garrod thus rose to the top of the medical profession without anyone much noticing. There were several reasons for this. His personality did not provide the stuff of anecdote; he attracted admiration but no idolatry; his clinical teaching was wonderfully informed but esoteric and therefore unlikely to inspire adulation by the general run of medical students; and, at Oxford, Osler was an impossible act to follow. Nor, despite Garrod's close friendships with the geneticist William Bateson and the biochemist Frederick Gowland Hopkins, did the scientific community follow his lead. The metabolic diseases Garrod studied were mostly rare and clinically unimportant. He appreciated their hereditary character, but he was uninterested in the developing science of mendelian genetics, and although he was

adept at the biochemical analysis of bodily fluids, he was not by nature an experimentalist. He was in essence a biologist in the clinic, at his best puzzling over diagnostic minutiae but less at home with routine treatment of common diseases.

In explaining Garrod's relative isolation in his own time, Alexander Bearn provides ample grounds for a modern appreciation of his subject. This admirable biography has two principal virtues. First, it is based on sound and extensive archival research. Bearn has followed Garrod to St Bartholomew's, to Oxford, to the Royal Society and other London institutions, and to Malta, where Garrod was stationed as a colonel in the Royal Army Medical Corps during the First World War. More importantly, he has uncovered and exploited Garrod's correspondence and other private papers still in the hands of his collateral descendants. This allows Bearn to offer a sympathetic account of Garrod's career, family life, friendships and self-image. It provides a moving gloss on his Oxford years and retirement; his life was shattered by the death of his two sons in the First World War and the loss of the third in the influenza pandemic of 1919. Only his daughter Dorothy survived, her distinguished career in archaeology partly stimulated by Garrod's own discovery of the subject during his years in Malta.

Of equal virtue is Bearn's eloquent analysis of Garrod's scientific ideas. Garrod's original four inborn errors of metabolism were alkaptonuria, albinism, cystinuria and pentosuria; he added a further two, haematoporphyrinuria and congenital steatorrhoea, in the second edition of his book. But for Bearn, Garrod's elaboration of the concept and methods of diagnosing inborn errors was not his most enduring intellectual contribution. Rather it was his notion of biochemical individuality, repeatedly invoked in his lectures and articles and the focus of his final book, *The Inborn Factors of Disease*, reluctantly published by Oxford University Press five years before his death. Bearn sees this as a foundation text of modern molecular medicine and human genetics and assesses the claim that the 'one gene, one enzyme' insight also originated from Garrod.

In his later years, Garrod became an apostle of what he liked to call the "higher medicine", the systematic application at the bedside of results gained in the laboratory. He practised what he preached even if those who listened were for the most part already converted. Bearn's volume is unlikely to turn his subject into a household name, but Garrod has been well served by his first biographer. □

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The chemistry of fullerenes

Roger Taylor & David R. M. Walton

Initially envisaged as rather unreactive, aromatic-like molecules, the fullerenes instead undergo a wide variety of reactions characteristic of alkenes. The many derivatives of C₆₀, and the few of C₇₀, that have now been reported offer new directions for organic chemistry.

JUST two and a half years ago the route to production of macroscopic amounts of C₆₀ and C₇₀ (Fig. 1) was described¹, to be followed rapidly by the method of chromatographic purification and proof of the structure². C₆₀ and C₇₀ are most accessible members of the family of closed-cage molecules known as fullerenes, formed entirely of carbon in the *sp*²-hybridized state, and constituting the third form of carbon (after diamond and graphite). The interest in these molecules can be gauged from the 1,300 or so papers that have already appeared. Most of these have described chemical physics aspects of the fullerenes, but the proportion devoted to their chemistry is growing and likely to become dominant in the years to come. Here we review the progress so far and consider possible future trends.

Each fullerene C_{*n*} consists of 12 pentagonal rings, and any number of hexagonal ones, *m*, such that *m* = (C_{*n*} - 20)/2 (Euler's theorem). Barely a year ago it was predicted that fullerenes containing seven-membered rings might exist³, and such is the speed of development in this field that they have already been found in carbon tubules⁴. When spectroscopic evidence for the existence of fullerenes was first obtained⁵, it was assumed that C₆₀ would be an extremely stable aromatic molecule. This view seemed to be supported by the calculation⁶ that there are 12,500 resonance structures possible for C₆₀. But this overlooks an important feature of strained five-(or four)-membered rings adjacent to benzenoid rings, namely the tendency to avoid having double bonds in the pentagonal (or tetragonal) ring. The presence of double bonds shortens bonds in the already-strained ring, producing the Mills-Nixon effect^{7,8}.

There is thus only one structure for C₆₀ that avoids having any double bonds in pentagonal rings, and this has two important consequences. First, delocalization of electrons is poor, so C₆₀ is much more reactive than originally expected. Otherwise C₆₀ would have all the chemical excitement of brick dust. Second, C₆₀ is not so much an aromatic molecule as a giant closed-cage alkene.

The same applies to other fullerenes. The need to avoid double bonds in the pentagonal rings largely governs the stability of fullerene derivatives, and hence the overall chemistry. It is also important in determining which fullerenes, and moreover which isomers, are stable. In general, stable fullerenes contain the structural characteristics of Fig. 2*a* whereas unstable fullerenes contain more of assemblies in Fig. 2*b* and *c* (ref. 9). The latter structure also introduces antiaromaticity, further contributing to the instability^{6,9,10}. These factors become less important for larger fullerenes as the destabilizing features will be a smaller proportion of the overall structure.

Unlike aromatics, fullerenes have no hydrogen atoms or other groups attached, and so are unable to undergo substitution reactions. Substitution reactions can take place on derivatives, once these have been formed by addition.

As the cages consist entirely of *sp*²-hybridized carbons, which have electron-withdrawing -I inductive effects¹¹, the fullerenes are strongly electron-attracting. This affects their chemical behaviour; for example they react readily with nucleophiles.

In summary, the molecules appear to undergo all the reactions associated with poorly-conjugated and electron-deficient alkenes (see Box 1). But their unique feature is the vast number of products that may arise from addition of just one reagent. The

chemistry of fullerenes is therefore concerned with investigating these additions, and examining the structures and reactions of the derivatives.

This is a considerable challenge. There can be a bewildering array of products, which mostly have poor solubility in common organic solvents, and this makes spectroscopic analysis of products difficult. The high cost and low availability of fullerenes necessitates working on a very small scale. Structural determination for such complex molecules cannot involve the degradative methods of classical organic chemistry, as the cages are unobliquely stable. Most derivatives fail to crystallize in a form suitable for single-crystal X-ray structure determination. Many derivatives have a great propensity (especially if they contain few adducts) to eliminate and reform the parent fullerene, making it difficult to determine structure by mass spectrometry; many derivatives give just the peak at 720 a.m.u. This caused the belief that isomers of C₆₀ were formed in flames. Derivatives that gave separate peaks were produced by high-pressure liquid chromatography, but just one peak at 720 a.m.u. by mass spectrometry¹². Two-dimensional ¹³C NMR methods have been successfully used for structural characterization (see for example ref. 13), but acquisition times of days on high-field instruments may be required. Fullerene chemistry is currently confined mainly to C₆₀, together with a little work on C₇₀. At present there is little prospect of chemistry on the higher fullerenes given that it takes about 250 hours of work to produce 1 mg of any of these.

Physical properties

Pure fullerenes have better close packing than impure fullerenes, and are therefore slower to dissolve²; the close packing evidently precludes their use as lubricants. C₆₀ and C₇₀ are decomposed by light, with oxygen and ozone playing a role¹⁴⁻¹⁶. The resultant degradation provided the first example of a fullerene cage-opening reaction¹⁴. Light-catalysed degradation occurs on alumina chromatography columns (especially of C₇₀; ref. 14), and our experience is that carbon/silica gel columns¹⁷ produce C₆₀ of a higher purity and crystallinity. Films of C₆₀ degrade on storage³, possibly because of the ability of C₆₀ to chemisorb oxygen (which increases its density)¹⁶. On heating in oxygen, C₆₀ is gradually oxidized: C-O adducts are formed at 200 °C, and at 400-500 °C decomposition is substantial¹⁸⁻²⁰.

Derivatives of C₆₀ show a wide range of solubilities; the fluorinated derivatives are much more soluble than the parent molecule, whereas some bromo derivatives are much less soluble.

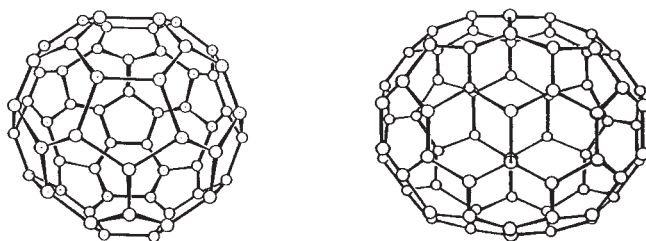


FIG. 1 C₆₀ and C₇₀.

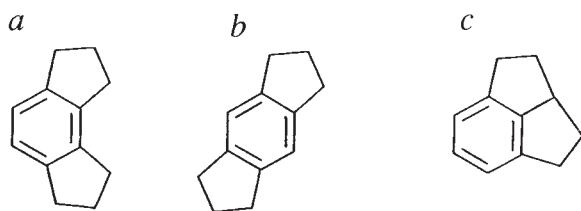


FIG. 2 The possible dispositions of two pentagonal rings adjacent to a hexagonal ring; dispositions *b* and *c* introduce instability.

This factor will be significant in choice of synthetic strategies involving fullerenes.

C_{60} molecules rotate rapidly in the crystal lattice ($\sim 10^{10}$ s⁻¹)^{21,22} but slow crystallization from benzene gives a solvate $C_{60}(C_6H_6)_4$ in which the rotation is slowed sufficiently to permit single-crystal X-ray determination of the cage structure^{23,24}. Similar results are obtained on crystallizing from benzene- CH_2I_2 (ref. 25) and from cyclohexane²⁶. Co-crystallization with benzene occurs in the complexes $(\eta^2-C_{60})Ir(CO)Cl(PPh_3)_2$ (ref. 27), $(\eta^2-C_{70})Ir(CO)Cl(PPh_3)_2$ where Ph represents phenyl, C_6H_5 (ref. 28) with toluene in $C_{60}O_2OsO_2(4\text{-}tert\text{-butylpyridine})_2$ (ref. 29), and with tetrahydrofuran in $(\eta^2-C_{60})Pt(PPh_3)_2$ (ref. 30). However, by incorporating the benzene rings into a ligand it is possible to obtain a host-guest complex, $(\eta^2-C_{60})Ir(CO)Cl(\text{benzyl-oxybenzyl}PPh_2)_2$ which crystallizes without incorporation of further solvent molecules³¹.

C_{60} can be encapsulated in the water-soluble host-guest complex $C_{60}(\gamma\text{-cyclodextrin})_2$ (refs 32, 33), and in the complex $[C_{60}(1,4\text{-hydroquinone})_3]^{34}$. C_{60} forms a charge-transfer complex with $[\text{bis}(\text{ethylenedithio})\text{tetrathiafulvene}]^{35}$. Co-crystallization with ferrocene produces $C_{60}(\text{ferrocene})_2$, a host-guest structure (Fig. 3)³⁶. Host-guest structures $C_{60}S_{16}$ and $C_{70}S_{48}$ (containing ordered fullerene) are formed from S_8 rings and C_{60} and C_{70} , respectively³⁷; the nucleophilicity of sulphur may be a contributing factor here.

The extent of charge separation in these complexes is important in relation to molecular conductors and soft ferromagnets, an area likely to attract continuing interest. Complexes could be important as a means of efficiently separating C_{60} and C_{70} , and so overcome the large losses of fullerenes (especially C_{70}) arising from irreversible absorption on the stationary phases in current chromatography.

Anion formation and oxidizing properties

A property which governs many of the chemical reactions of C_{60} and C_{70} is that up to six electrons can be added, reversibly, to each³⁸⁻⁴⁰. Because delocalization of electrons is poor in the fullerenes, one may account for the additions in terms of localized structures. Adding one electron to each pyracyclene unit creates aromaticity in one of the component pentagonal rings (Fig. 4; compare with ref. 41). There are six such pyracyclene units in C_{60} , arranged octahedrally (Fig. 5a), and a driving force for octahedral addition is the creation of eight isolated benzenoid rings (in which delocalization constraints are removed)⁴². There are also six pyracyclene units in C_{70} (Fig. 5). Six pyracyclene units are also present in both D_2 and D_{2d} isomers, which are the main components of C_{84} (refs 43-45), and the reversible addition of five electrons to C_{84} has so far been detected by cyclic voltammetry⁴⁶.

It follows that these fullerenes are oxidizing agents, and for example C_{60} catalyses the oxidation of H_2S to sulphur⁴⁷. Likewise both molecules should be readily reduced, so C_{60} undergoes reduction under Birch conditions⁴⁸, by hydrogen with Pt catalyst (D. K. Heimbach *et al.*, unpublished work), diimide (R. Roers, *et al.*, unpublished work), hydrogen transfer reagents such as dihydrophenanthrene⁴⁹, and by diborane in THF (which gives

$C_{60}H_2$)⁵⁰. Cyclic voltammetry profiles 38-40 indicate that addition to C_{70} may be easier than to C_{60} , and that the hexaanion at least is more stable for C_{70} . C_{70} may therefore be the better oxidizing agent, and be more readily reduced. Thus C_{70} forms $C_{70}H_{12}$, probably through addition across the interpentagonal bonds in each pyracyclene unit⁴⁵.

Coordination of Ni, Pd and Pt Ligands to C_{60} produces a decrease in the number of reversible reduction waves (those centred on the C_{60} -framework), and the potential of the first reduction step, due to the ligand electron-donating character⁵¹.

C_{76} shows different electrochemical behaviour, giving four reduction waves and one oxidation wave in cyclic voltammetry⁵². It should therefore behave as both an oxidizing agent and a reducing agent. Its electron-donating potential may have consequences for its reactivity, and also mean it can be separated from other fullerenes through the use of electron-acceptor solvents.

Anions prepared by reduction of C_{60} with lithium metal react with methyl iodide to produce a mixture of polymethylated derivatives mainly containing an even number of methyl groups⁵³. Up to 24 methyl groups are added, with the six- and eight-methyl derivatives dominant (compare this with the halogenation and phenylation results below).

More controlled anion formation has been achieved in the reaction of C_{60} with *t*-butyl-lithium^{54,55}. This yielded $t\text{-Bu}C_{60}^-$ (where Bu is butyl, C_4H_9), together with small amounts of $(t\text{-Bu})_n C_{60}^{n-}$ ($n=2-6$) which with acid gave the corresponding hydrogenated derivatives. $t\text{-Bu}HC_{60}$ exists in two forms, with the H and *t*-Bu groups in either the 1,2- or 1,11-positions (see ref. 56 for nomenclature). The latter rearranges into the former which is more stable, despite being eclipsed, because there is no double bond in a pentagonal ring. There are no other forms, because delocalization of the electrons would place double bonds in pentagonal rings, and this factor also inhibits hydrogen migration over the cage surface. The high electron withdrawal by the

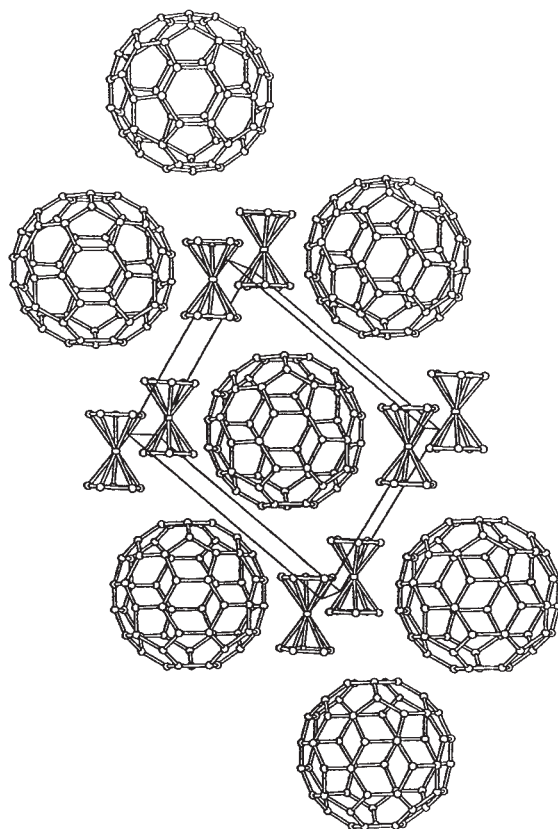


FIG. 3 Host-guest structure of $C_{60}(\text{ferrocene})_2$, which is probably stabilized by weak intermolecular charge-transfer interactions.

cage (coupled possibly with a strong eclipsing interaction between the adjacent addends) makes *t*-BuHC₆₀ a very strong carbon acid ($pK_a = 5.7$), with a weak (71 kcal mol^{-1}) C–H bond dissociation energy. High acidity of the C₆₀–H bond may account for the solubility, in the basic aqueous layer, of the products of LiAlH₄ reduction of fluorinated C₆₀ (ref. 57). The high acidity of hydrogenated derivatives may lead to some unique applications. The acidity should decrease with increasing level of reduction, so that it may be possible to use a pH-dependent regime to separate the various hydrogenated derivatives.

Addition reactions

Addition to fullerenes presents unusual problems. The rigidity of the cage means that eclipsing interactions can hardly be avoided, except, by introducing C–C bond strain^{58,59}. It is therefore difficult to obtain stable derivatives in which bulky groups lie adjacent, and the addition pattern changes according to the size of the added groups. Additions can be categorized into cycloadditions, additions involving bridging (here there is no steric hindrance) and additions of separate groups.

Cycloadditions. The electron-withdrawing nature of fullerenes makes them ideal dienophiles for Diels–Alder (2+4) cycloadditions, which takes place readily⁴¹. Because of the ease of adduct loss, identification of the products by mass spectrometry is difficult, but can be simplified by reduction of the addend, thereby preventing the retro-reaction. In this way six cyclopentadienes have been shown to add to C₆₀, indicating that reaction

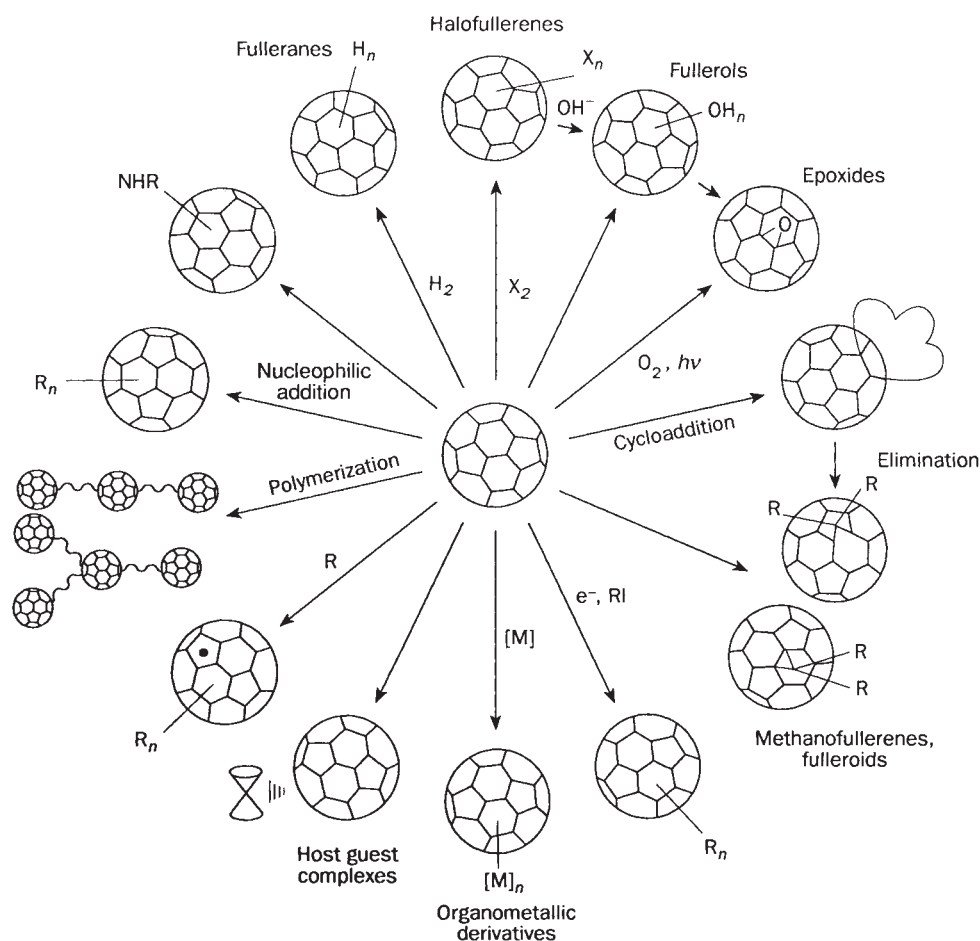
occurs across each pyracyclene unit⁶⁰. Success has also been achieved using adducts which by losing carbon monoxide, gain aromaticity and are then unable to return to the starting materials (see Fig. 6)^{61,62}.

A similar approach has led to a successful (2+2) cycloaddition using benzyne, which is converted upon addition to benzene, thereby inhibiting the reverse reaction. Up to six benzyne molecules add to C₆₀ (refs 63, 64), and it should also be possible to add up to six benzyne to C₇₀ without encountering steric problems. The derivatives should undergo electrophilic substitution at the β -aromatic position, thereby paralleling the behaviour of benzocyclobutene⁶⁵.

Ultraviolet irradiation of C₆₀ in the absence of oxygen gives a polymeric product (C₆₀)_n (*n* up to 20), almost certainly as a result of photochemically allowed (2+2) cycloaddition⁶⁶. Four-membered rings are probably produced (as in the reaction with benzyne), and have the potential to undergo the usual ring-opening reactions of strained aliphatic rings, so providing a valuable route to 1,2-addition products. Previously the ultraviolet irradiation of fullerenes has been described as producing coalescence⁶⁷.

Addition involving bridging. *Oxygen bridges.* There have been many reports of the formation of epoxides^{63,68–72}, with C₆₀ being isolated and characterized^{73,74}. Oxygen can also be more firmly bound in the form of ethers produced either by ultraviolet irradiation¹⁴ or by heating the epoxides¹⁶; extensive irradiation degrades these to carbonyl-containing compounds^{14,75}. With

BOX 1 Reactions of C₆₀.
SOME general reactions known to occur with C₆₀ (and to a lesser extent with C₇₀).



dimethyldioxirane, C_{60} either abstracts oxygen to form the epoxide, or forms a 1,3-dioxolane derivative⁷⁴.

A good example of binding to oxygen is the reaction of fluorinated C_{60} with aqueous methanol, whereby up to 18 oxygens (believed to be present as epoxides) become attached to the cage⁷⁶. In $C_{60}O$, the epoxide is formed across the 6,6 central bond of a pyracylene unit (Fig. 7a, where X is O). However, theoretical calculations indicate that the (less-strained) oxidoannulene structure (Fig. 7b, X is O), should be the more stable^{77,78}. $C_{70}O$ is predicted to have an oxidoannulene structure at the waist of the molecule⁷⁸.

Carbon bridges. Methylene adducts of C_{60} and C_{70} , were first observed by mass spectrometry⁶⁸. Ar_2C groups (Ar is an aromatic ring) derived from aryldiazomethane precursors have been added across one of the 6,6 bonds of C_{60} . Up to six of these groups can be added, suggesting an octahedral array^{41,79,80}. The structure is now known to be a bridged one analogous to that for the epoxide (Fig. 7a, where X is CAr_2) (F. Wudl, personal communication). By contrast, addition of CH_2 by this method takes place across a 5,6-ring junction giving a fulleroid (Fig. 7b, where X is CH_2)⁴¹. Aryl-substituted methanofullerenes might be developed into carbon-chain polymers^{80,81}, whereas those with amino or hydroxy groups in the aromatic rings might yield fullerene-containing polyamides and polyesters.

Methanofullerenes have also been prepared wherein the methano group forms part of a glucose moiety⁸².

Metallic bridges. In reaction with metal derivatives, C_{60} also behaves as an alkene (giving η^2 coordination) rather than as an aromatic (which would require η^6 coordination)³⁰. By suitable choice of conditions, either one, two or six platinum groups can be added, the latter giving an octahedral array³⁰. Addition of platinum (and also of osmium tetroxide, below) has shown how a second group may add to C_{60} . Assuming that addition occurs across the 6,6-bond of high order, then eight bonds are available for addition of the second group. Three of the isomeric di-adducts were detected, but surprisingly, two of these are not on the direct pathway to the octahedral compound, suggesting that adduct migration (possibly by a dissociation-reassociation mechanism) must accompany addition⁸³. Monoadducts have also been formed with nickel and palladium⁷³.

Iridium complexes give stable η^2 adducts with electron

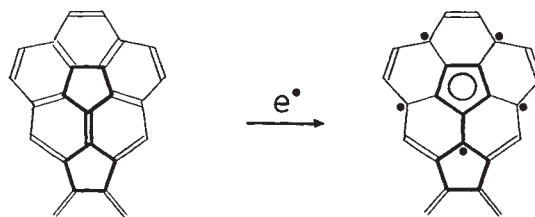


FIG. 4 Addition of an electron to a pyracylene unit of a fullerene to give an aromatic pentagonal ring.

deficient alkenes, and consequently add across a 6,6-ring fusion site in C_{60} to give crystalline derivatives^{27,84}. Two iridium adducts (with $PPhMe_2$ ligands, where Me is methyl, CH_3) have been attached to C_{60} at exactly opposite sides of the cage⁸⁵. An interesting feature here is that the phenyl groups can point either towards the cage or away from it, yielding in principle three isomers: endo, exo; endo, endo; exo, exo, and the last two have been isolated and characterized. $Ir(CO)Cl(PPh_3)_2$ also adds across one of the high-order π -bonds adjacent to the pentagonal cap in C_{70} (Fig. 8)⁵⁸. Two iridium adducts containing $PPhMe_2$ ligands have also been attached to C_{70} (ref. 86). Each adds across an a-b bond² adjacent to each of the polar caps. Three positional isomers are possible, namely the 1,9:61,62; 1,9:63,64; and 1,9:67,68 derivatives (see ref. 56 for numbering system), and the product is the former of these in which all phenyl groups are endo⁸⁶. This isomer must also be chiral, as also should be the 1,9:67,68 derivative. There are fifteen possible isomers arising from a combination of positional, chirality, and exo, endo factors for addition of these particular iridium adducts adjacent to the polar caps of C_{70} .

Osmylation. We have already described metallic derivatives involving formation of a three-membered ring. With osmium tetroxide/4-*t*-butylpyridine, addition typical of an alkene again occurs to give an osmate ester (Fig. 9)^{29,87}. Five isomeric di-adducts have been obtained, two of which involve addition at octahedral sites¹³.

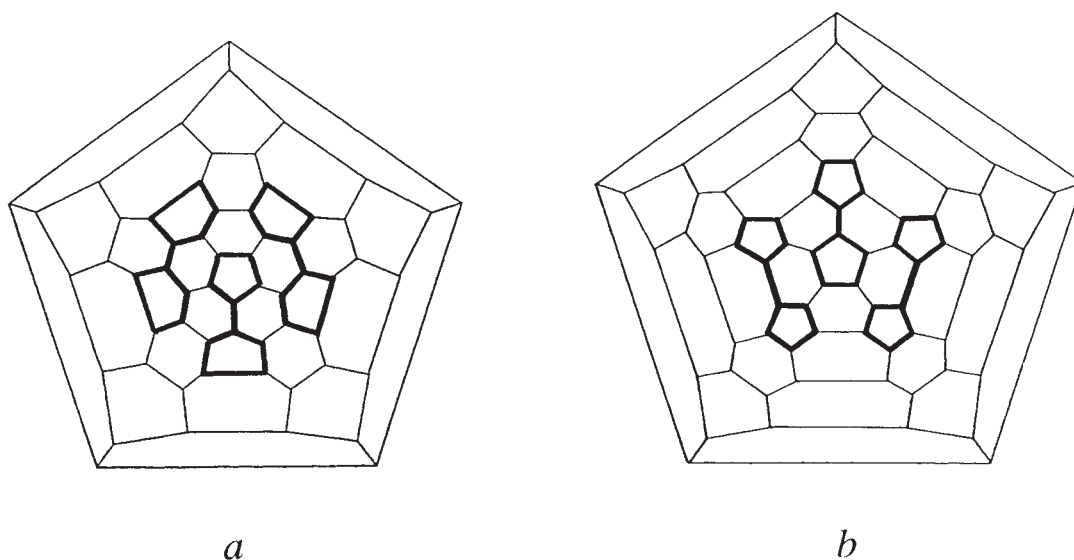


FIG. 5 Schlegel diagrams for (a) C_{60} and (b) C_{70} showing three pyracylene units outlined for each molecule. Three other units are in each molecule located symmetrically with respect to those outlined.

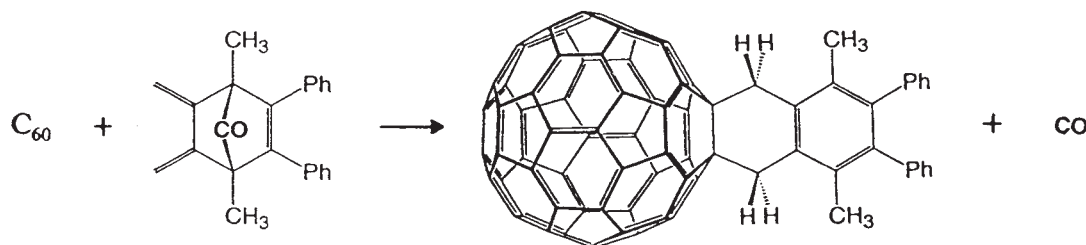


FIG. 6 Reaction of C_{60} with 5,6-dimethylene-1,4-dimethyl-2,3-diphenylnorborn-2-en-7-one, giving a product unable to undergo retro-Diels-Alder elimination.

Addition of separate groups. Addition of halogens and hydrogen.

Only 24 groups can be added to C_{60} without any two being adjacent (Fig. 10), and this is the maximum number that has been reported in methylation⁵³, chlorination^{88,89} and bromination^{90,91}. The single-crystal X-ray structure of $C_{60}Br_{24}$ confirmed these latter dispositions⁹¹, which were also predicted⁹⁰ from the structure of $C_{60}Br_8$ (encircled area, Fig. 10), obtained from different brominating conditions. Other conditions yielded $C_{60}Br_6$ (Fig. 11)⁹⁰; each bromo derivative is solvated by approximately two bromine molecules. The halogeno compounds decompose to C_{60} on heating and the stability order is chloro > bromo, and $C_{60}Br_{24} > C_{60}Br_8 > C_{60}Br_6$, the latter reverting first to $C_{60}Br_8$ (ref. 90). The thermal instability of these compounds prevents their mass spectra being obtained. $C_{60}Cl_6$ has also been prepared and its structure shown by ^{13}C NMR to be analogous to that of $C_{60}Br_6$ (ref. 92).

By contrast, the addition of up to sixty fluorines to C_{60} has been reported⁹³ but the stability of the fluoro derivatives decreased beyond about $C_{60}F_{40}$ (ref. 94) making subsequent addition of fluorine very slow⁹³. The fluoro compounds are more stable than the chloro and bromo derivatives but decompose rapidly on heating above 80 °C (R. T., unpublished results) and also on storage at room temperature in C_6F_6 (ref. 72). The more highly fluorinated species are less stable, so that peaks for $C_{60}F_{50}$, $C_{60}F_{60}$ in mass spectrum disappear after sublimation⁹⁵. C_{70} seems to fluorinate more readily than C_{60} (A. Brisden, J. H. Holloway & E. C. Hope, personal communication) and as fluorine is very slow to penetrate the fullerene lattice, this probably reflects poorer packing in C_{70} than in C_{60} ; fluorinated C_{70} seems to be less stable than fluorinated C_{60} (ref. 72). Fluorofullerenes are stable enough to give mass spectra, and $C_{60}F_{60}$ has now been detected in this way⁹⁵, although it is probable that the mass peak intensities do not accurately reflect the product composition.

As halogeno derivatives are key intermediates in organic syntheses, bromination in this area is likely to be intense. At present, fullerene bromination is the most controlled halogenation, but

gives the most insoluble derivatives. By contrast the fluoro derivatives are very soluble and reactive (see below), but their formation (necessarily under heterogeneous conditions) is difficult to control.

It might have been expected that many hydrogenated derivatives of C_{60} would by now have been isolated and characterized. But although reduction is easy, the derivatives are intractable, change their solubility on standing and, disconcertingly, show the presence of oxygen-containing derivatives in the infrared. The products after standing seem to be acidic, and appreciably soluble only in polar solvents such as dimethyl-sulphoxide, pyridine and aqueous acetone (D. K. Heimbach, *et al.*, unpublished results). Allylic autoxidation probably occurs, and because singlet oxygen (of which C_{60} is a potent producer⁷⁵) usually accounts for this process⁹⁶, C_{60} may catalyse autoxidation of its hydrides. Evidence has been obtained for the formation (under Birch reduction conditions) of $C_{60}H_{18}$ and $C_{60}H_{36}$ (ref. 48) and $C_{60}H_2$ (which is unstable in the presence of C_{60} ; P. A. Cahill, personal communication) has been isolated⁵⁰; such a species may be strongly acidic⁹⁷.

Reaction with electrophiles. The electron-withdrawing nature of the cages makes them unreactive towards electrophiles, but this obstacle is overcome in their anions. Thus methylated derivatives are produced by reaction of C_{60} anions with methyl iodide. Both $(CH_3)_6$ and $(CH_3)_8$ derivatives predominate in the products, which contained up to 24 methyl groups⁵³. This is somewhat similar to results for bromination (and also phenylation, below).

Addition of nucleophiles. C_{60} reacts readily with both neutral and charged nucleophiles. An example of the former is the reaction with a wide range of amines (up to 12 amine molecules can be added), giving acid-soluble products⁹⁷. The chief products contain six amino adducts⁹⁸ again implying an octahedral array resulting from addition across the 6,6 bond of each pyracylene unit. The temperature-dependent proton NMR signal of the product was initially interpreted in terms of hydrogens migrating

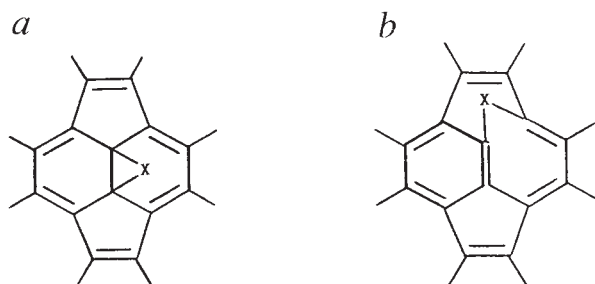


FIG. 7 a, Formation across the 6,6 bond of a C_{60} pyracylene unit of epoxide (where X is O) and cyclopropane rings (X is CAr_2); the latter derivative is a 61,61-diarylmethano-1,2-fullerene-60. b, Insertion into a 5,6 bond of C_{60} of either oxygen (X is O, 'oxidoannulene', predicted) or methylene (X is CH_2 , 1,6-fulleroid-60, observed).

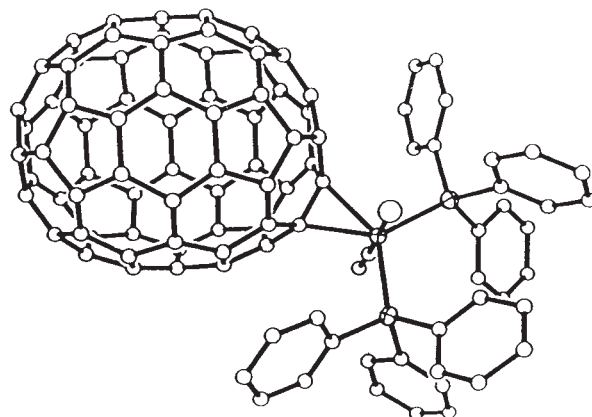


FIG. 8 Adduct of C_{70} with $Ir(CO)Cl(PPh_3)_3$.

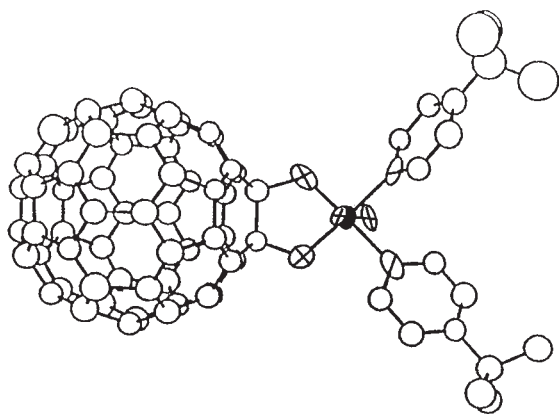


FIG. 9 Adduct of C_{60} with osmium tetroxide/4-t-butylpyridine: ●, osmium; ⊕, oxygen; ⊙, nitrogen.

over the surface of the cage. However, this would require the unfavourable introduction of double bonds into pentagonal rings, and the NMR signals have since been shown to arise from water present in the amine; similar results can be obtained using other amines⁹⁹. Reaction with morpholine gives the product $C_{60}H_6(\text{morpholine})_6$ which undergoes hydrogen exchange with CH_3OD , again indicating the additivity of the C_{60} -hydrogen bond, although cyclic voltammetry indicated that the bond is covalent⁹⁸.

Reaction with charged nucleophiles is rapid, as demonstrated in alkylation and arylation by either Grignard or organolithium reagents followed by reaction with methyl iodide. In this way, mixtures of derivatives containing components such as $C_{60}Me_{10}Ph_{10}$, $C_{60}Me_3Ph_2$, and $C_{60}Me_7Bu$ have been obtained⁹⁷, and the monoalkylated derivatives $C_{60}H_7Bu$ and $C_{60}HEt$ (Et represents ethyl, C_2H_5) have also been isolated^{54,55}.

In these reactions there is electrophilic displacement of the metallic group by C_{60} . More familiar electrophilic behaviour is seen in the reaction of C_{60} with aromatics in the presence of either $AlCl_3$ or $FeCl_3$ ^{89,100}. The reactions are thought⁸⁹ to take place through initial protonation of the cage by the $HAICl_3OH$ super acid. A variety of products $C_{60}H_nAr_n$ have been obtained, especially $n=12$ and 16 when the aromatic group is Ph, significant in view of nucleophilic substitution results described below.

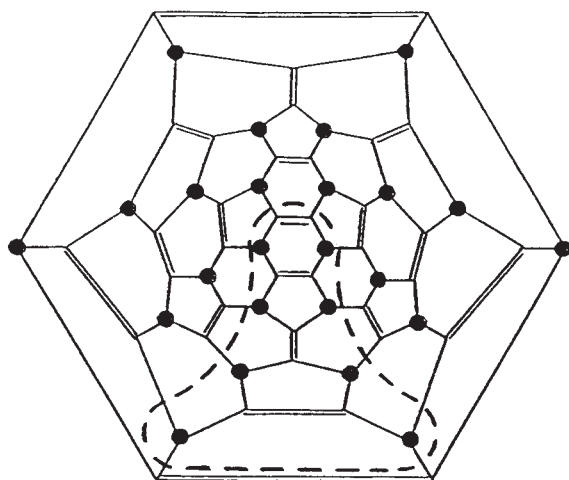


FIG. 10 Schlegel diagram showing the 24 non-adjacent sites in C_{60} . These are the locations of the bromine atoms in $C_{60}Br_{24}$; encircled sites show the locations of the bromines in $C_{60}Br_8$.

Hydroxy groups can also be added to C_{60} by reaction with diborane/THF followed by $H_2O_2/NaOH$ (N. Schneider *et al.*, unpublished results). The infrared spectrum of the product is virtually identical to that obtained by reaction with nitrating reagents/NaOH (refs 101, 102) and likewise is surprisingly more soluble in acid than in water. A band in the spectrum at $\sim 1,590\text{ cm}^{-1}$ may be due to a hemiketal structure (Fig. 12); treatment of the fullerol with acid causes this band to diminish, and a carbonyl band at $\sim 1,725\text{ cm}^{-1}$ appears^{101,102}. A mechanism for this (an example of fullerene cage-opening) and also an acid-induced pinacol rearrangement have been deduced (Fig. 12)^{101,102}.

The fullerols produced by the nitration regime contain about 18 hydroxy groups. Formation of a nitro intermediate by electrophilic addition of nitronium ion followed by nucleophilic replacement by OH has been proposed for the mechanism involving mixed acid. But fullerols are produced (albeit less readily) even if sulphuric acid alone is used, and it may be that NO_2-OH and $H-OH$ adducts are intermediates, as are NO_2-OCOR adducts in the similar reaction with nitronium tetrafluoroborate/carboxylic acids^{101,102}. These reactions are likely to be studied intensively, given the water-solubility of the products and the possibility of developing cage-opening reactions.

Addition of radicals. The radicals $Me\cdot$, $Ph\cdot$, $PhS\cdot$, $PhCH_2\cdot$, $CBr_3\cdot$, $CCl_3\cdot$, $CF_3\cdot$ and $Me_3CO\cdot$ react readily with C_{60} , although in some cases rapid reversion to C_{60} occurs^{103,104}. Up to 11 phenyl groups, 15 benzyl groups and 34 methyl groups have been added by means of radical reactions^{103,105}. The radicals $C_{60}(\text{benzyl})_n$, $n=3, 5$ (Fig. 13) are particularly stable, and are highly localized as extensive delocalization would mean putting double bonds in pentagonal rings. Further, the structures of the pentabenzyl radical and $C_{60}Br_6$ are remarkably similar. This suggests that $C_{60}Br_5\cdot$ may lie on the pathway to formation of the latter, but cannot be effectively stabilized by hyperconjugation, and so acquires a further bromine atom. By contrast, in the benzyl radicals the enforced alignment of the sigma C-C bond parallel to the p -orbitals of the cage creates a perfect opportunity for C-C hyperconjugation⁹⁰. (For the general importance of the latter see ref. 106.)

In radicals $C_{60}R\cdot$, the group R is believed to be located at a 6,6 bond, delocalization of the radical being restricted to the *ortho*- and *para* positions in the adjacent hexagonal ring^{104,107}. The evidence from electron spin resonance is that the $C_{60}t\text{-Bu}$ radical forms a dimer, and steric constraints suggest that dimeri-

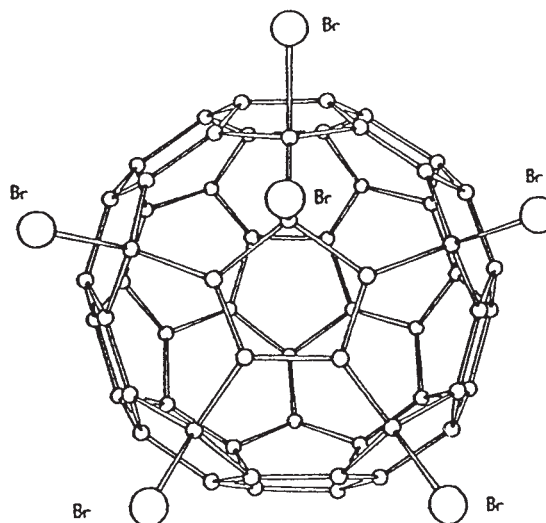


FIG. 11 Structure of $C_{60}Br_6$.

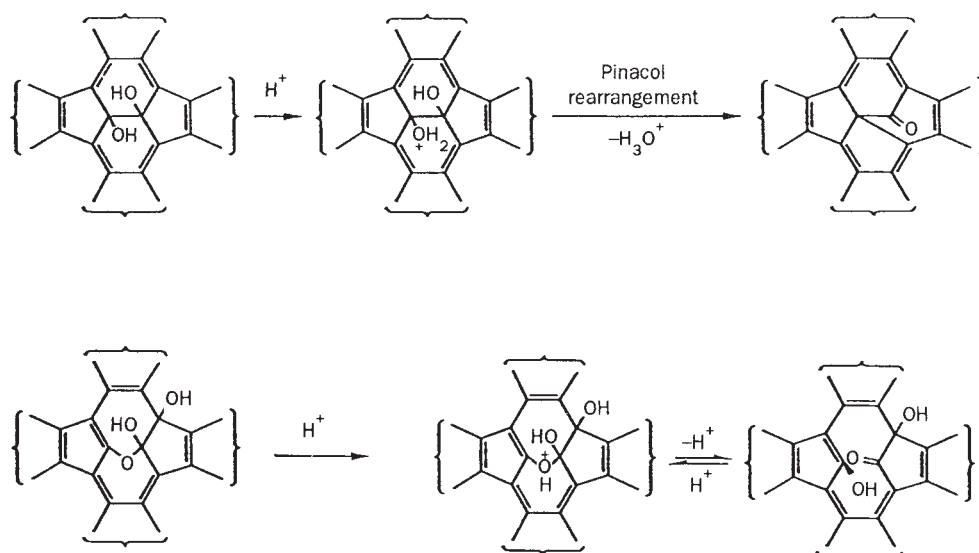


FIG. 12 Proposed effect of acid on fullerols.

zation must take place at the *para* position. $C_{60}CCl_3\cdot$ and $C_{60}CBr_3\cdot$ are believed also to dimerize¹⁰⁴. Radicals containing smaller alkyl groups are probably not dimer-driven, as there will be less steric hindrance towards further addition.

Three different radicals $C_{70}R\cdot$ have been detected¹⁰⁸, and these may be related to the three different interpentagonal ring junctions in C_{70} (Fig. 5). These are the general a, b and c carbons² or the specific 1-, 6- and 7-carbons⁵⁶. So far, occupancy of the first of these has been positively identified.

Nucleophilic and electrophilic substitution

Nucleophilic substitution of substituted fullerenes may also be defined (in reaction with aromatics) as electrophilic substitution by the fullerene derivative (or by the fullerene itself). These reactions are likely to attract considerable research effort given that many of the products are themselves intermediates for extensive further chemistry.

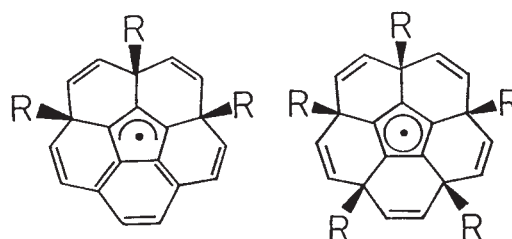
Just as the parent fullerenes are reactive towards nucleophilic addition, so the derivatives are very reactive towards nucleophilic substitution. Fluorinated C_{60} reacts readily with nucleophiles including water, evolving HF which precludes the use of these materials as lubricants¹⁰⁵. With very reactive nucleophiles such as amines the reactions are instantaneous, whereas with weak nucleophiles such as acetic acid, substitution requires many weeks. Preliminary work in which fluorines have been replaced by carbon, oxygen or nitrogen nucleophiles, as well as by hydride ion, indicates that the rate of nucleophilic substitution decreases as more fluorines become replaced⁵⁷. Chlorinated C_{60} reacts with sodium methoxide giving products up to $C_{60}(OMe)_{34}$ which exhibit a broad envelope of methoxy groups centred at 3.7 p.p.m. in proton NMR⁸⁹. A similar NMR pattern is observed in reaction of the fluoro compounds with $NaOCH_3/CH_3OH$, some sharp singlets indicating the presence of symmetrical products⁵⁷, and these singlets also appear in the product of reaction of $NaOCH_3/CH_3OH$ with brominated C_{60} (P. R. Birkett, *et al.*, unpublished data). The order of reactivity of the halogeno derivatives of C_{60} seems to be fluoro > chloro > bromo, and this may reflect either homogeneity effects (solubilities decrease along this series) or a nucleophilic substitution in which the first step is rate-determining. Because backside attack is impossible in these compounds, the reactions may involve an addition-elimination mechanism⁵⁷.

The reaction of halogenated derivatives of C_{60} with aromatics in the presence of Lewis acid catalysts is a classic example of an electrophilic aromatic substitution, where the halogenated C_{60} is the electrophile. Reaction of chlorinated C_{60} with benzene in the

presence of $AlCl_3$ gave products containing up to 22 phenyl groups⁸⁹. Reaction of C_{60} with bromine, benzene and $FeCl_3$ gave a product consisting of $C_{60}Ph_n$ with values of n up to 16. The species with $n=12$, $n=8$ and $n=6$ especially prominent⁷¹ (see ref. 100). The predominance of the latter species is significant in the light of the results for bromination and methylation, described above. The mechanism of the reaction seems to involve preformation of brominated C_{60} species which are the reaction electrophiles. Similar substitutions into toluene occur.

Polymerization

Polymers involving C_{60} may be of three types: two of these are 'pearl necklace' (Fig. 14a) and 'pendant chain' (Fig. 14b) polymers¹¹⁰. We propose that two- and three-dimensional variants of the former should be described as 'net' and 'lattice' polymers. The third type, a necklace variant with direct links between the cages is apparently formed on polymerization of C_{60} by ultra-violet irradiation in the absence of oxygen⁶⁶. This technique may be adaptable to derivatives, although stereo control of the reaction would be difficult. C_{60} has been incorporated in a copolymer with *p*-xylylene (1:3.4 molar ratio), but the product (expected to be of the necklace type) is unstable and reacts fairly readily with oxygen¹¹¹. C_{60} has been incorporated into non-cross-linked polystyrene by reaction with $AlCl_3$, giving a product apparently highly cross-linked⁸⁸. Anionic copolymerization of C_{60} with styrene monomer gives a mixture of products, termed flagellanes (lattice type), which are highly soluble and can be processed in the melt; as such they may be either spin-coated, solvent-cast, or melt extruded¹¹². The average molar ratio of

FIG. 13 Structure of allylic and cyclopentadienyl radicals formed from C_{60} .

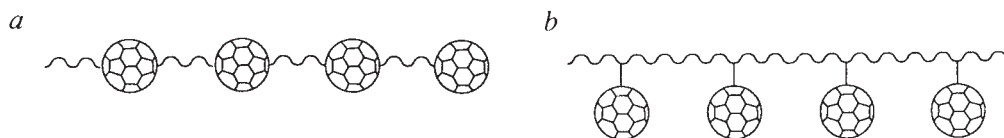


FIG. 14 'Pearl necklace' and 'pendant chain' polymers that in principle can be made with fullerenes; the circle represents the fullerene cage.

styrene to C_{60} in the product is ~ 20 , and $\sim 4 \times 10$ chains are accommodated on the fullerene core¹¹², consistent with the expected value of six. These polymers are thus of the lattice type.

Hydroxy and amino-substituted arylmethanofullerenes⁴¹ have the potential for production of C_{60} -containing polyesters and polyamides, and the former may in principle also be obtained from fullerenols^{101,102}. The methanofullerenes can give rise to pendant chain polymers^{80,81}.

Reaction between C_{60} and the complex Pd_2 -(dibenzylideneacetone)₃CHCl₃ results in ligand displacement to give $C_{60}Pd_n$ (ref. 113). Larger values of n were obtained if larger stoichiometric amounts of the complex were used, but were never less than 1.0 if larger amounts of C_{60} were used. The complex $C_{60}Pd$ is believed to consist of a linear chain of palladiums bonded each to two C_{60} molecules. When this complex is heated, C_{60} is regenerated and the value of n increases to ~ 3.0 , consistent with the formation of a lattice polymer. Such materials may have important applications, given that $C_{60}Pd_{3.5}$ (although not $C_{60}Pd_n$, $n < 3$) catalysed the hydrogenation of diphenylacetylene, and this is consistent with the presence of palladium atoms on the polymer surface.

Future directions

The extraordinary structure of fullerenes, and of C_{60} in particular, has fuelled the expectation that they will find unique applications. In consequence, many patents concerning fullerenes and derivatives have been filed worldwide. Some possibilities have been noted here; C_{60} also shows promise as an optical limiter¹¹⁴, as an enhancer of photoconducting properties of some polymers¹¹⁵ and (in the form of hydrides) as a material for high specific capacity batteries¹¹⁶.

Chemically, C_{60} behaves typically for an electron-deficient alkene, but there are three aspects that may be the focus of future research. First, the tendency of some derivatives to revert readily to C_{60} , coupled with the cage bulk, suggests their use for stereoselective transfer of addends, for example in bromination. Second, the greater solubility of derivatives suggests that for those containing few addends, it may be possible to have the solubility benefit, without losing a particular physical property of the parent fullerene. Third, the extraordinary facility for adding oxygen, which is not yet understood, may find many uses, and lead to a separate field of fullerene chemistry.

The nucleophilic substitution of fullerene derivatives may need special attention, given that a new mechanism seems probable: of the usual mechanisms, the S_N1 mechanism is ruled out by the strong electron withdrawal by the cage, and S_N2 is sterically precluded.

Lastly, the difficulties associated with the purification of fullerenes, assessment of purity and separation of the numerous reaction products may stimulate the development of analytical and separation techniques, to the lasting benefit of all chemistry. This may, in the end, be the fullerenes' most valuable legacy. □

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Crystallographic analysis of the catalytic mechanism of haloalkane dehalogenase

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Crystal structures of haloalkane dehalogenase were determined in the presence of the substrate 1,2-dichloroethane. At pH 5 and 4 °C, substrate is bound in the active site without being converted; warming to room temperature causes the substrate's carbon–chlorine bond to be broken, producing a chloride ion with concomitant alkylation of the active-site residue Asp₁₂₄. At pH 6 and room temperature the alkylated enzyme is hydrolysed by a water molecule activated by the His₂₈₉–Asp₂₆₀ pair in the active site. These results show that catalysis by the dehalogenase proceeds by a two-step mechanism involving an ester intermediate covalently bound at Asp₁₂₄.

THE nitrogen-fixing hydrogen bacterium *Xanthobacter autotrophicus* GJ10 can grow on a medium containing 1,2-dichloroethane or 2-chloroethanol as the sole carbon and energy source¹. The organism is being investigated for application in the clean up of environmentally harmful halogenated compounds, some of which are industrially produced in large amounts for use as cleaning agents, pesticides and solvents. The first step in the degradation of these compounds by the bacterium is catalysed by haloalkane dehalogenase¹, an enzyme having an *M_r* of 36K and a known nucleotide and amino-acid sequence^{2,3}. It converts 1-haloalkanes into primary alcohols and a halide ion by hydrolytic cleavage of the carbon–halogen bond, with water as a co-substrate and without any need for oxygen or cofactors², at an optimal pH of 8.2. The crystal structure of the enzyme has been determined at 2.4 Å resolution⁴ and refined to 1.9 Å (ref. 27).

The putative active site is located between the two domains of the protein in an internal, predominantly hydrophobic cavity. Asp₁₂₄, His₂₈₉ and Asp₂₆₀, which are located in this cavity, have been proposed to be the catalytic residues. They form a catalytic triad, with the triad residues occurring at the same topological positions as in other members of the α/β hydrolase-fold family⁵, despite any clear sequence homology. To this family also belong acetylcholinesterase from *Torpedo californica*⁶, diene lactone hydrolase from *Pseudomonas* sp. B13 (ref. 7), carboxypeptidase II from wheat^{8,9} and lipases¹⁰. All members of this family have a similar core formed by an α/β sheet of eight β -strands connected by α -helices. In addition, they all have a small pocket near the amide nitrogen of the residue following the nucleophile, which could be the oxyanion hole⁵, indicating that they may share a similar reaction mechanism. The proposed nucleophilic residue for the dehalogenase (Asp₁₂₄) is quite different from that of the other enzymes (Ser or Cys), however, and the bond to be

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hydrolysed (carbon-halogen) is rather different from the ester or amide bond cleaved by the other enzymes. From the architecture of the active site of dehalogenase, there are two possible reaction mechanisms for hydrolytic dehalogenation: the first could be a nucleophilic substitution by the carboxylate anion of Asp₁₂₄, resulting in a covalently bound intermediate ester that might subsequently be hydrolysed by a water molecule, activated by His₂₈₉ as base (Fig. 1a)^{4,11}; the second possibility is a general base catalysis with a water molecule activated by His₂₈₉ (ref. 11; Fig. 1b).

To discriminate between these two possibilities and to compare the dehalogenase catalytic mechanism with that of the other members of the α/β hydrolase-fold family, we analysed the catalytic pathway in dehalogenase as a function of pH and temperature by high-resolution X-ray crystallography. Both putative dehalogenase catalytic mechanisms require that the His₂₈₉ side chain is mainly deprotonated at the pH of optimal activity. A crystal structure determined at pH 8.2 shows that this is indeed the case^{5,27}. But at low pH, with the His₂₈₉ side chain protonated and no longer acting as a base, according to the first mechanism the covalently bound intermediate could accumulate in the active site, whereas by the second mechanism, substrate should bind without being degraded. We therefore soaked dehalogenase crystals in mother liquor containing the substrate 1,2-dichloroethane at different pHs and temperatures. This allowed us to obtain three-dimensional structures of (1) dehalogenase with substrate bound in its active site; (2) dehalogenase with the covalently bound intermediate; (3) dehalogenase with chloride as the product of the reaction bound in the active site. From these structures we conclude that the reaction catalysed by the dehalogenase proceeds through a two-step catalytic mechanism with a covalently bound intermediate.

Substrate binding

Substrate was bound in the active site of dehalogenase by soaking crystals for three hours in mother liquor¹² with 10 mM 1,2-dichloroethane at pH 5.0 and 4 °C. A difference Fourier showed clear density in the active-site cavity in which a 1,2-dichloroethane molecule could be fitted. The final structure, refined at 2.4 Å resolution, shows that one chlorine atom of the substrate (Cl-1) is bound to the ring nitrogen atoms of Trp₁₂₅ and Trp₁₇₅ (Cl-N distances are 3.6 Å and 3.2 Å, respectively) (Fig. 2a). Cl-1 lies nearly on the intersection of the planes of the two tryptophan rings, thus maximizing the favourable interactions with the slightly positively charged hydrogen atoms of the aromatic ring nitrogens. The second chlorine atom (Cl-2) is not bound very tightly and may be flexible, interacting with the side chains of Phe₁₂₈, Phe₁₇₂ and perhaps Phe₁₆₄. The substrate's C₁ carbon

atom, which is connected to Cl-1, is near the O_{δ1} atom of Asp₁₂₄, at 3.8 Å. The protein atom closest to C₂ is C_{γ2} of Val₂₂₆, at 3.3 Å; the Asp₁₂₄ O_{δ1} atom is at 3.6 Å. At pH 6.2 as well as under the present conditions, the Asp₁₂₄ side chain is hydrogen-bonded to the N_{ε2} atom of His₂₈₉ (distance is 2.6–2.7 Å). At

TABLE 1 Summary of the crystallographic analyses

	Crystal 1 pH 5.0 + 10 mM substrate + cooling at 4 °C	Crystal 2 pH 5.0 + 10 mM substrate + RT	Crystal 3 pH 6.2 + 10 mM substrate + RT
pH	5.0	5.0	6.2
Temperature	4 °C	22 °C	22 °C
Number of crystals	1	1	1
Soaking time	3 h	24 h	96 h
Total exposure time less	48 h	48 h	48 h
Resolution range	2.39 Å	1.99 Å	2.14 Å
Space group	<i>P</i> 2 ₁ 2 ₁ 2	<i>P</i> 2 ₁ 2 ₁ 2	<i>P</i> 2 ₁ 2 ₁ 2
Unit cell dimensions:			
<i>a</i>	94.9 Å	94.8 Å	95.0 Å
<i>b</i>	72.8 Å	72.8 Å	72.6 Å
<i>c</i>	41.4 Å	41.4 Å	41.4 Å
Total number of observations	34,590	39,634	36,467
Number of unique reflections	11,326	16,812	14,119
Number of discarded observations	2,549	1,254	1,284
<i>R</i> _{merge} (%)	12.44	4.06	5.74
Completeness of the data (%)	94.6	82.0	84.7
Stereochemical quality			
Number of residues	1–310	1–310	1–310
Number of solvent molecules	125	151	188
Final <i>R</i> -factor (%)	19.5	18.6	16.5
Coordinate error estimates from a σ_A plot (Read, 1986)	0.12 Å	0.15 Å	0.13 Å
R.m.s. deviations from ideality for bond lengths (Å)	0.014	0.010	0.009
bond angles	3.9°	3.0°	2.9°
Special features compared to native dehalogenase:	Substrate bound in active-site cavity	Alkyl-enzyme Chloride product	Chloride product Hydrolytic water has disappeared New incoming water in cavity
	Hydrolytic water	Hydrolytic water disappears	

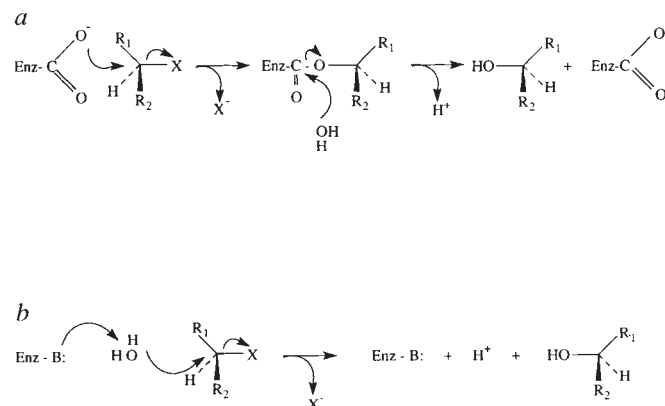


FIG. 1 a, b, Possible reaction mechanisms for the hydrolytic dehalogenation by haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10.

RT, room temperature. The gene coding for native haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 was cloned and overexpressed in cultures of *Escherichia coli* HB101 (ref. 3). The isolated and purified enzyme^{2,3,27} gave good sized crystals grown from a 60% (w/v) saturated (at 0 °C) (NH₄)₂SO₄ solution buffered at pH 6.2 with 100 mM bis-Tris(bis(2-hydroxyethyl)-iminotris(hydroxymethyl)-methane), using vapour diffusion in hanging drops¹². The space group is orthorhombic, *P*2₁2₁2, with unit cell dimensions *a* = 94.8 Å, *b* = 72.8 Å and *c* = 41.4 Å. Soaking of the crystals in mother liquor containing 10 mM 1,2-dichloroethane at the different pHs and temperatures had negligible effect on the size of the unit cell. X-ray intensities of the different complexes were collected with a FAST area detector (Enraf Nonius, Delft) equipped with a CAD4 κ -goniostat, with graphite monochromatized CuK α radiation from an Elliot GX21 rotating anode generator as X-ray source. Data collection and reduction was done with the MADNES system²²; profile fitting of the data was according to Kabsch²³. Data were scaled²⁴ and merged using the Groningen BIOMOL crystallographic software package.

$$R_{\text{merge}} = \frac{\sum_{hkl} \sum_{\text{refl}} |I(hkl, j) - \bar{I}(hkl)|}{\sum_{hkl} \sum_{\text{refl}} I(hkl, j)} \times 100\%$$

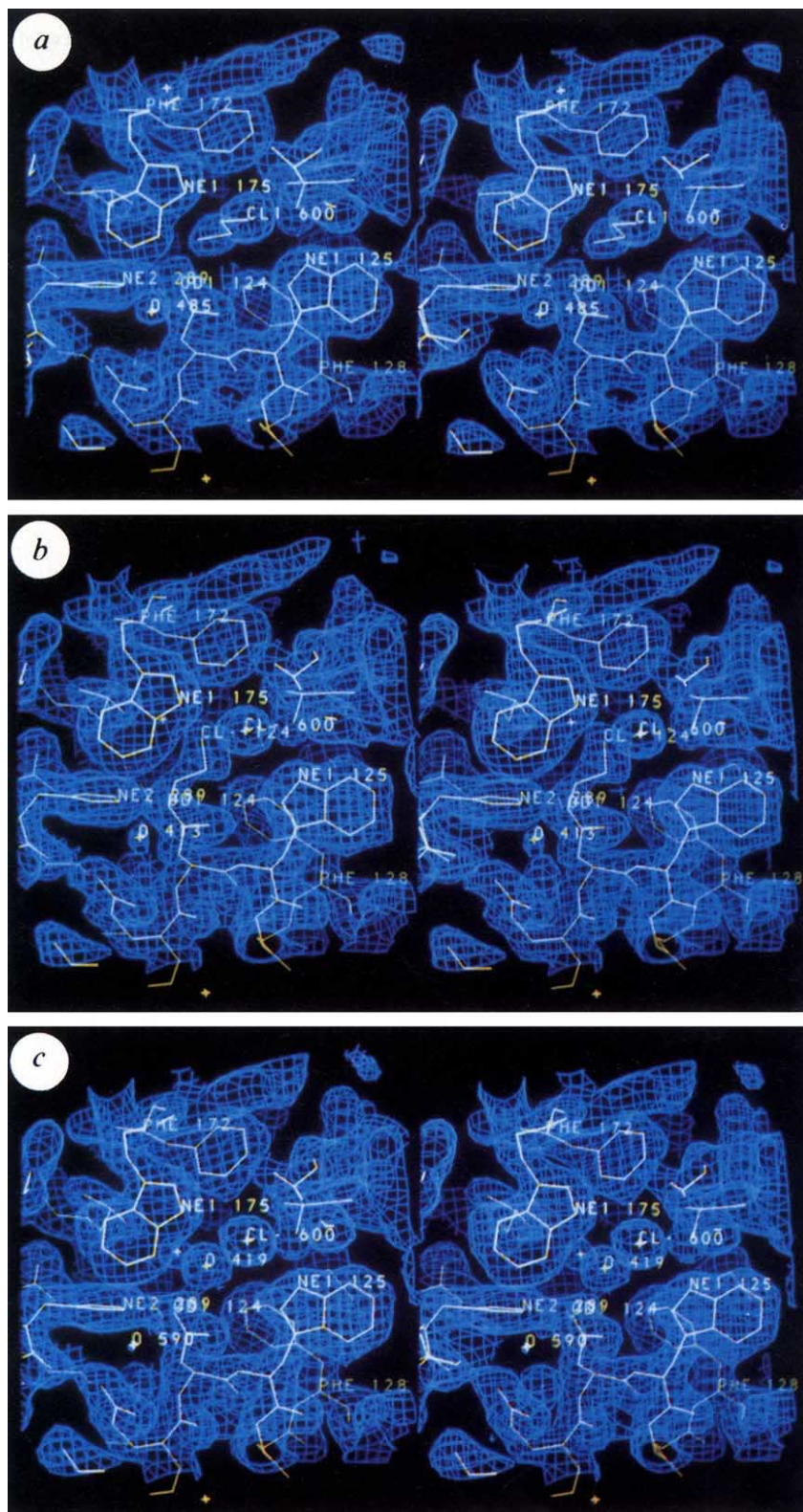
where *I* is the observed intensity and $\bar{I}$ is the average intensity obtained from multiple observations of symmetry-related reflections.

$$R\text{-factor} = \frac{\sum |F_{\text{obs}}| - |F_{\text{calc}}|}{\sum |F_{\text{obs}}|} \times 100\%$$

pH 8.2, the pH optimum of the dehalogenase, this hydrogen bond is lost²⁷ and both the Asp₁₂₄ and His₂₈₉ side chains have moved apart (by 0.6 Å). Assuming that the substrate position at pH 8.2 is the same as at pH 5.0, the Asp₁₂₄ O_{δ1} atom at pH 8.2 would be somewhat closer to the substrate's C₁ atom (at about 3.7 Å), and could perform a nucleophilic attack. There are no water molecules close to one of the substrate's carbon atoms, the nearest water molecule being at 5.7 Å from C₁. This makes

a direct attack of a water molecule on the C₁ carbon atom of the substrate extremely unlikely. However, there is a water molecule (H₂O₄₈₅ in Fig. 2a) present close to the C_γ atom of Asp₁₂₄ (distance 2.8 Å), which could hydrolyse the proposed ester intermediate at Asp₁₂₄. This water molecule is near the N_{ε2} atom of residue His₂₈₉, which also hydrogen-bonds with its N_{δ1} atom to the side chain of Asp₂₆₀. A schematic view of the residues important for substrate binding is given in Fig. 3a.

FIG. 2a Stereo view of the final 2.4 Å-resolution σ_A -weighted²⁵ $(2m|F_{\text{obs}}| - D|F_{\text{calc}}|)\exp(i\alpha_{\text{calc}})$ electron density map showing a substrate molecule bound in the active site of haloalkane dehalogenase. Crystals were mounted in X-ray capillaries at 4°C and directly transferred to a FAST television area detector, where data collection was carried out at 4°C over a two-day period. The pH 6.2 structure of dehalogenase, determined at 1.9 Å resolution²⁷ without solvent molecules, was used as the starting model for the crystallographic refinement with the TNT program²⁶. Details of the soaking procedure, data collection and the refinement statistics are given in Table 1. b, Stereo view of the final 2.0 Å-resolution σ_A -weighted²⁵ $(2m|F_{\text{obs}}| - D|F_{\text{calc}}|)\exp(i\alpha_{\text{calc}})$ electron density map showing the electron density for the covalently bound intermediate of the dehalogenase. c, Stereo view of the final 2.14 Å-resolution σ_A -weighted²⁵ $(2m|F_{\text{obs}}| - D|F_{\text{calc}}|)\exp(i\alpha_{\text{calc}})$ electron density map showing the electron density for the dehalogenase with chloride as a reaction product bound. The atomic coordinates have been deposited with the Brookhaven Protein Data Bank.



The alkyl-enzyme

Soaking native dehalogenase crystals for one day in 10 mM 1,2-dichloroethane at pH 5 and at room temperature caused the accumulation of a covalent intermediate in the crystal (Fig. 2*b*). Clear extra density extends from the O_{δ1} atom of the side chain of Asp₁₂₄, and the covalently bound intermediate can easily be built into this density. A spherical electron density, consistent with the density for a chloride ion in an ($|F_{\text{obs}}|(\text{substrate}) - |F_{\text{obs}}|(\text{native})$) difference Fourier is located between the side chains of two tryptophan residues (residues 125 and 175). This density is not connected to the density extending from the Asp₁₂₄ side chain, indicating that the carbon-halogen bond has been cleaved, and that a chloride ion and a covalently bound intermediate have been formed. This is clear evidence for the proposed two-step mechanism, with the Asp₁₂₄ side chain acting as the first nucleophile⁴. The chloride ion is at the same position as the Cl-1 atom in the bound substrate, and interacts with the ring nitrogen atoms of the Trp residues (at 3.5 Å and 3.2 Å from the N_ε atoms of Trp₁₂₅ and Trp₁₇₅, respectively). The two carbon atoms of the covalently bound substrate make van der Waals contacts with Phe₁₂₈, Trp₁₇₅ and His₂₈₉. In contrast to the weak density observed for the Cl-2 atom in the enzyme-substrate complex, the density for Cl-2 in the alkyl-enzyme complex is unambiguous (Fig. 2*b*). Cl-2 lies in the plane of the Phe₁₇₂ side chain and may be stabilized by interactions with the hydrogen atoms at the C_{δ1} and C_{ε1} atoms of this residue. The Asp₁₂₄ and His₂₈₉ side chains have barely moved relative to their position in the enzyme-substrate complex. The carbonyl oxygen of the ester intermediate (Asp₁₂₄ O_{δ2} in the free enzyme) is hydrogen-bonded to the peptide nitrogen atoms of residues 56 and 125, whereas the ester oxygen atom (Asp₁₂₄ O_{δ1}) is hydrogen-bonded to the His₂₈₉ N_{ε2} atom. Also the water molecule close to the C_γ atom of Asp₁₂₄ in the complex with substrate, is still present, although its electron density has decreased (H₂O₄₁₃ in Fig. 2*b*). Assuming the temperature factor of this water to be similar to those of the surrounding atoms, its occupancy refined to about

0.4. This strongly suggests that, under our experimental conditions, this water molecule is slowly acting as the nucleophile in the second reaction step, in which the ester intermediate is hydrolysed, without being replenished. This water is completely secluded from the solvent and it is probably replenished from the active site cavity (see below). Some new electron density is already observed for an incoming water molecule near the Cl-2 atom. In agreement with the partial occupancy of the hydrolytic water, we estimate the occupancy of the covalently bound intermediate to also be about 0.4 to 0.5. Figure 3*b* gives a schematic overview of the interactions of the alkyl group with the enzyme.

After hydrolysis of the alkyl intermediate

Soaking native dehalogenase crystals for two days at room temperature in a mother liquor with 10 mM 1,2-dichloroethane at pH 6.2 results in a complex of dehalogenase with chloride as the product (Fig. 2*c*). There is no longer any density corresponding to the covalently bound intermediate, or the product 2-chloroethanol. But we still observe a spherical density consistent with a chloride ion located between the side chains of Trp₁₂₅ and Trp₁₇₅ (Fig. 2*c*). Also an ($|F_{\text{obs}}|(\text{product}) - |F_{\text{obs}}|(\text{native})$) difference Fourier clearly discriminates this chloride ion from a solvent molecule. Thus, at pH 6.2 the crystallized enzyme has low activity. One of the products of the reaction, the 2-chloroethanol, has already left the active site cavity, but the chloride ion—the other product—is still present. In addition to the chloride ion, the active-site cavity contains two water molecules. One occupies a position near where the Cl-2 atom was situated in the covalently bound intermediate, and the other is hydrogen-bonded to the O_{δ1} atom of the Asp₁₂₄ side chain (Fig. 3*c*). The O_{δ1} atom is also hydrogen-bonded to the His₂₈₉ N_{ε2} atom, as we found in the native dehalogenase structure at pH 6.2 (refs 4, 27). To our surprise, although the density of the hydrolytic water molecule is clear near the C_γ atom of Asp₁₂₄ in the native structure at pH 6.2 and in the enzyme-substrate complex and the alkyl-enzyme, it has almost completely disappeared in the

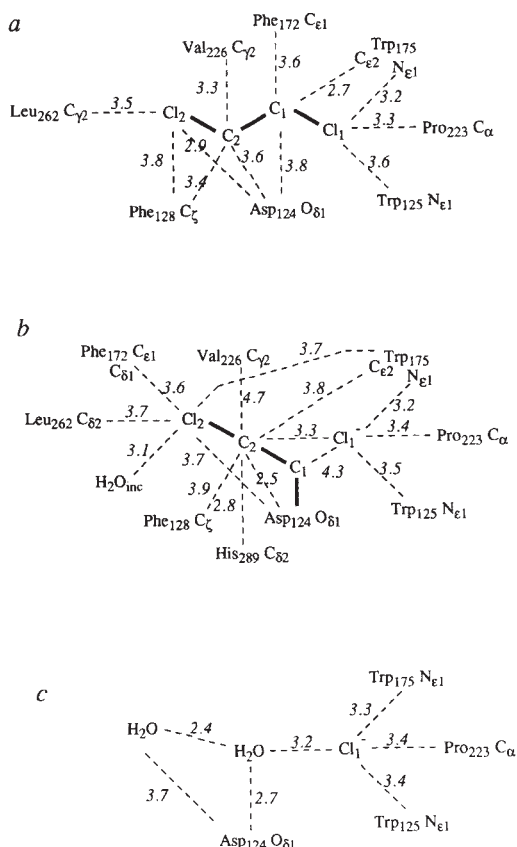


FIG. 3 Schematic view of the interactions of the active site residues of dehalogenase *a*, with the substrate, before the start of the reaction; *b*, with the alkyl intermediate and the chloride ion, during the reaction; *c*, with the chloride ion and the water molecules, after hydrolysis.

present structure (H_2O_{590} in Fig. 2c). This indicates that this is the water molecule essential for the hydrolysis of the covalently bound intermediate, but that it is replenished after hydrolysing the alkyl-enzyme only very slowly under the conditions applied. The water molecule most likely to replenish the hydrolytic water is the one hydrogen-bonded to the Asp₁₂₄ side chain. Presumably

at pH 6.2, the hydrogen bond between the Asp₁₂₄ and His₂₈₉ side chains is strong enough to limit the mobility of Asp₁₂₄ and to restrict the accessibility to the binding site of the hydrolytic water molecule. Indeed, if the experiment is done at pH 8.2, we find that the hydrolytic water molecule has been (partly) replenished. Interestingly, in contrast to the native dehalogenase structure at

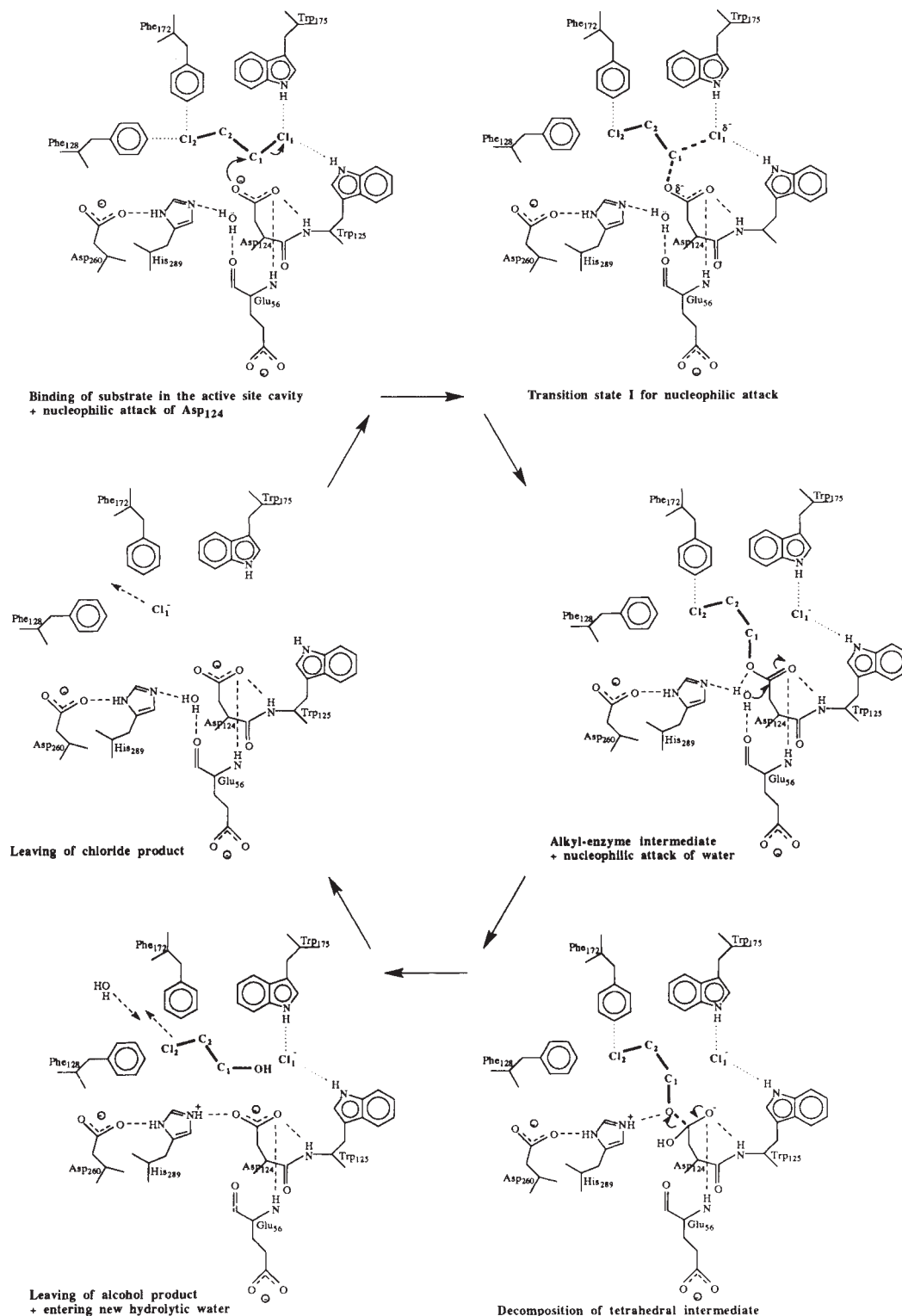


FIG. 4 Reaction mechanism at the optimal pH (pH 8.2) of haloalkane dehalogenase as deduced from the crystal structures of the native

enzyme without substrate at pH 8.2 (ref. 27) and from the structures described here.

pH 8.2, in which there is no hydrogen bond between the Asp₁₂₄ and His₂₈₉ side chains²⁷, a hydrogen bond exists between Asp₁₂₄ and His₂₈₉ even at pH 8.2 if a chloride ion is present in the active site. This suggests that the release of chloride from the substrate increases the pK_a of the His₂₈₉ side chain, thereby facilitating its function as a general base to activate the hydrolytic water molecule.

Discussion

By variation of pH and temperature in soaking experiments of native haloalkane dehalogenase crystals with the substrate 1,2-dichloroethane, we succeeded in trapping different stages in the enzyme's reaction pathway. The first step of the reaction, the formation of the enzyme-substrate complex, could be trapped at low pH and low temperature. Increasing the temperature resulted in the accumulation of a covalently bound intermediate, and increasing, in addition, the pH to 6.2 allowed the release of the alcohol product but left the chloride product still bound in the active site. The reaction intermediates that we observe are reasonably stable under the experimental conditions. Their lifetimes are long enough to allow collection of diffraction data sets by standard methods over two-day periods without completely being converted. The resulting structures show the intermediates in significant occupancies.

Our experiments confirm the two-step catalytic mechanism proposed earlier⁴, in which Asp₁₂₄ is the nucleophile in the first step of the reaction and a water molecule hydrolyses the covalent ester intermediate in the second step (see Fig. 4 for a summary of the reaction mechanism). In this respect they are consistent with the reaction mechanisms in other α/β hydrolase-fold enzymes, in which a covalent intermediate is also formed that is hydrolysed by a water molecule (for example, acetylcholinesterase¹³ and serine carboxypeptidases¹⁴). But whereas in these other enzymes the same carbonyl carbon atom of the substrate molecule is attacked twice (first by the enzyme's nucleophile (Ser or Cys), and subsequently by a water molecule), in dehalogenase two different atoms are targets of nucleophilic attack; first the C₁ carbon atom of the substrate is attacked by the O_{δ1} atom of Asp₁₂₄ to form a covalently bound ester, and next this ester is hydrolysed by a water molecule that attacks the C_γ atom of the Asp₁₂₄ residue. As a consequence, the dehalogenase reaction pathway proceeds through completely different transition states, each with its own stabilization. In the first step, the transition state is presumably a penta-coordinated C₁ carbon atom of the substrate, as occurs in S_N2 substitution reactions at sp³ carbon atoms, with the negative charge developing on the halogen atom stabilized by the slightly positively charged N-H atoms of the Trp₁₂₅ and Trp₁₇₅ side chains. This step is unique for the dehalogenase. The second reaction step proceeds through

a tetrahedral intermediate at the C_γ atom of Asp₁₂₄. The transition states for the formation and breakdown of this tetrahedral intermediate may be stabilized by interactions with the peptide nitrogen atoms of Glu₅₆ and Trp₁₂₅, which are in the small oxyanion pocket. The O_{δ2} atom of Asp₁₂₄, which is negatively charged in the tetrahedral intermediate, hydrogen-bonds with these backbone NH groups in all the native and complexed dehalogenase structures analysed so far. All α/β -fold hydrolases have such a small pocket in common, near the amide nitrogen of the residue following the nucleophile⁵. In the wheat serine carboxypeptidase, the peptide nitrogen of Gly₅₃ was suggested to be part of the oxyanion-binding site⁹. It is located at the C terminus of β -strand 3 (α/β hydrolase-fold numbering) at a position equivalent to that of Glu₅₆ in dehalogenase. In addition, in dehalogenase the O_{δ2} atom of Asp₁₂₄ is positioned very close to the axis of helix C, at the N-terminal side of the helix. Thus, the negative charge developing on the O_{δ2} atom may be further stabilized by the α -helix dipole^{15,16}. β -strand 5, the nucleophilic residue, and helix C form the so-called nucleophile elbow, which is the most conserved structure in all α/β hydrolases⁵. Therefore it seems likely that the method of stabilization of the tetrahedral intermediate is the same for all α/β hydrolases, with conservation of the actual mechanism for the hydrolysis of the intermediate ester. In agreement with this is the observation that the interatomic interactions in the active sites are very similar. Dehalogenase extracts a proton from the hydrolytic water molecule at the N_{ε2} atom of the active-site histidine, while Asp₂₆₀ interacts with the N_{δ1} atom of His₂₈₉ through a *syn*-type hydrogen bond. Identical interactions are present in the active sites of diene lactone hydrolase⁷, serine carboxypeptidase⁹, *Geotrichum candidum* lipase¹⁰ and acetylcholinesterase⁶, although these last two enzymes have a glutamic acid interacting with the histidine instead of an aspartic acid residue. Moreover, in the members of the α/β hydrolases with an Asp-His interaction, the plane of the Asp side chain makes an angle of ~45° with the plane of the imidazole ring, unlike in the serine proteases, in which the carboxylate side chains are nearly coplanar with the imidazole ring^{17,18}.

Normally, the lifetime of reaction intermediates in wild-type enzymes is too short for crystallographic observation and millisecond Laue crystallography^{19,20} or site-directed mutagenesis²¹ have to be used. Here, the combination of low pH and low temperature, and the disparity between alkylation and dealkylation mechanisms in the dehalogenase, with dealkylation as the rate-limiting step under our conditions, allowed us to accumulate different reaction intermediates in the crystals and to analyse these intermediates by conventional protein crystallographic methods. We hope to clarify further details of the reaction mechanism using appropriate mutations. □

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Laboratory evidence for ionized polycyclic aromatic hydrocarbons in the interstellar medium

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THE origin of the broad interstellar infrared emission bands (at 3.3, 6.2, 7.8, 8.6 and 11.3 μm) found in the vicinity of many galactic and extragalactic sources is still poorly understood. The original suggestion¹ that the bands are associated with aromatic species embedded in small carbon particles was later challenged by the proposal² that they originate from vapour-phase, neutral polycyclic aromatic hydrocarbons (PAHs); Allamandola *et al.* independently argued³ that PAH cations are the source of the bands. This latter proposal has steadily gained acceptance, but the lack of experimentally determined emission spectra of PAH cations has made it difficult to test the idea. We have recently measured the visible and infrared spectra of the neutrals and cations of four PAHs — naphthalene⁴, anthracene⁵, pyrene⁶ and perylene⁷ — dispersed in argon matrices at 12 K, to approximate the low-pressure, gas-phase conditions of the interstellar medium. Here we compare the infrared absorption from these four molecules (neutrals and cations), integrated over the spectral regions corresponding to the interstellar bands, with the astronomical observations. We find that the interstellar bands cannot be explained solely on the basis of neutral PAH species, but that cations must be a significant, and in some cases dominant, component.

The PAH cations were produced^{4,5} by low-energy (50–70 eV) electron impact of a vapour-phase mixture of the sublimed PAH, Ar and CCl_4 (0.2%). The addition of CCl_4 to the mixture is important both for increasing the number of ions via Penning ionization (with Ar^*) and charge transfer (with Ar^+) and for providing electron traps (Cl , CCl_3^+ and so on) in the matrix. The irradiated mixture was deposited on a cold (12 K) BaF_2 window held on the cold finger of a closed-cycle helium cryostat (ADP, Displex). After deposition the matrix was scanned in the visible and ultraviolet regions (Cary 17 spectrophotometer) to note the presence and abundance of the cationic species. PAH cations are known to absorb in the visible range⁸, whereas the neutrals generally absorb in the ultraviolet⁹. With a simple 90° turn of the cryostat, the infrared spectrum was next determined (Nicolet 7199 Fourier-transform infrared spectrometer (FTIR), 300 scans, 1 cm^{-1} resolution). The FTIR detector was positioned

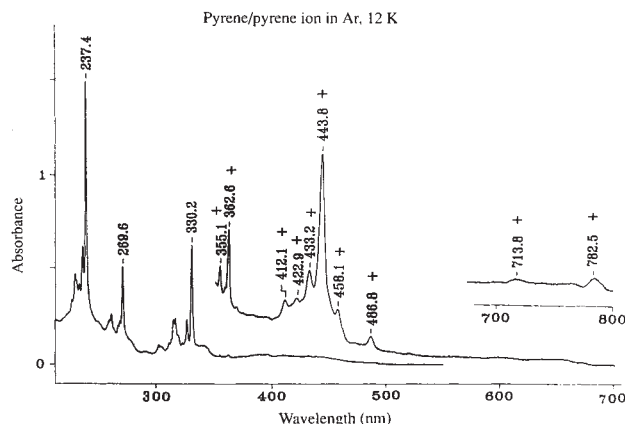


FIG. 1 Visible and ultraviolet absorption spectra of neutral pyrene and cationic pyrene (band marked by crosses) in Ar matrix at 12 K.

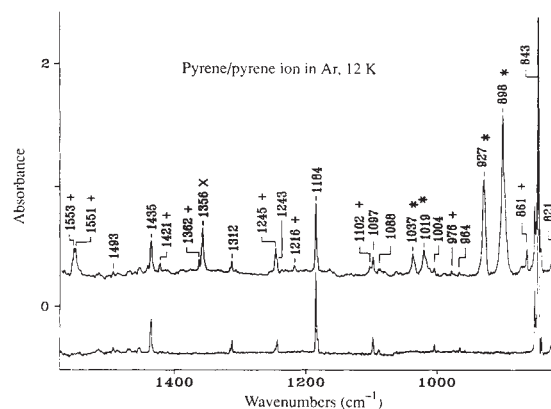


FIG. 2 Infrared spectra of neutral pyrene (lower trace) and mixture of neutral and cationic pyrene (upper) in Ar matrix at 12 K. Pyrene cation bands are marked by crosses, CCl_4 bombardment products by stars.

below the Cary 17 sample compartment with the appropriate optical steering mirrors and N_2 purging system. The infrared band positions and relative intensities of the new cation bands were checked and found to be independent of the CCl_4 added to the matrix mixture. Correlation between the intensities of the

TABLE 1
Frequencies and integral intensities of infrared absorption bands

	$\nu(\text{cm}^{-1})$	$\lambda(\mu\text{m})$	Integral intensity (km mol^{-1})*
Naphthalene ⁴	1,525	6.56	31.2 (0.16)
	1,519	6.58	15.6 (0.08)
	1,401	7.14	7.8 (0.04)
	1,218, 1,215	8.22	195.0 (1.00)
	1,023, 1,016	9.8	50.7 (0.26)
Anthracene ⁵	1,540	6.49	7.3 (0.04)
	1,457	6.86	9.1 (0.05)
	1,418	7.05	176.8 (0.97)
	1,410	7.09	16.4 (0.09)
	1,341	7.45	182.3 (1.00)
	1,291	7.75	12.8 (0.07)
	1,188	8.42	178.6 (0.98)
	1,034	9.67	36.4 (0.20)
	912	10.96	27.3 (0.15)
Perylene ⁷	432	23.15	5.5 (0.03)
	1,567	6.38	39.9 (0.18)
	1,551	6.44	108.7 (0.49)
	1,396	7.16	33.3 (0.15)
	1,349	7.41	221.8 (1.00)
	1,318, 1,316	7.58	104.3 (0.47)
	1,313	7.61	37.7 (0.17)
	1,230	8.13	26.6 (0.12)
	1,221	8.19	13.3 (0.06)
	1,211	8.26	13.0 (0.06)
Pyrene ⁶	1,203, 1,200	8.33	20.0 (0.09)
	1,128	8.86	26.6 (0.12)
	819	12.21	97.6 (0.44)
	750.5	13.33	141.9 (0.66)
	1,553, 1,551	6.44	169.3 (1.00)
	1,440	6.94	11.8 (0.07)
	1,421	7.04	20.3 (0.12)
	1,362, 1,358	7.34	60.0 (0.35)
	1,245	8.03	74.5 (0.44)
	1,216	8.22	16.9 (0.10)
	1,102	9.07	11.8 (0.07)
	976	10.25	8.5 (0.05)
	861	11.61	66.0 (0.39)
	690	14.49	32.2 (0.19)

Samples in Ar matrix, 12 K.

*Relative intensities given in parentheses.

TABLE 2 Integral Intensities of some PAHs and their cations in regions of interstellar interest

Neutral/cation	$I(11.3\mu\text{m})$ 810–925 cm^{-1} (km mol^{-1})	$I(7.8+8.6\mu\text{m})$ 1,030–1,400 cm^{-1} (km mol^{-1})	$I(6.2\mu\text{m})$ 1,525–1,630 cm^{-1} (km mol^{-1})	$I(3.3\mu\text{m})^\dagger$ 3,000–3,100 cm^{-1} (km mol^{-1})
Naphthalene/Naph cation	1.4/*	20/195	6/31	50/
Anthracene/An cation	98/27	35/408	22/7	80/
Perylene/Pe cation	100/97	52/497	17/149	60/
Pyrene/Py cation	155/66	52/163	20/169	87/
Sums	354/190	159/1263	65/356	277/

The left-hand entries in each column are the integrated intensities within the designated energy range for the neutral PAHs, and the right-hand entries are for the cations of the PAHs.

* No bands observed for the cation in this region; value for neutral species is low because the main C–H band occurs at 788 cm^{-1} ($12.7\mu\text{m}$), outside the indicated region.

† No C–H stretch bands due to the cations observed in this region.

visible bands due to the cations and the new infrared bands was sought by varying the abundance of the cation produced; we did this by varying the electron beam energies, irradiation (medium-pressure Hg lamp) and deposition durations, and PAH/Ar/ CCl_4 ratio. Figures 1 and 2 show results for one of the PAHs, pyrene (Py). The visible spectrum of the Py cation (Fig. 1) closely resembles one reported for Py^+ in a neon matrix¹⁰. The infrared spectra of neutral Py and a mixture of neutral and cationic Py in argon are shown in Fig. 2. Finally Fig. 3 shows the positive correlation plots linking the 443.8-nm visible band and the 976 , 1358 , 1362 , and $1553/1551\text{ cm}^{-1}$ infrared bands (correlation coefficient $r > 0.95$). For one newly observed infrared band ($1,356\text{ cm}^{-1}$), the above correlation was poor ($r < 0.8$), and we consider that this band does not belong to Py^+ .

The integrated intensities of the infrared bands were obtained as follows^{4,5}. Knowing the molar absorptivity of an ultraviolet band of the neutral PAH and assuming that the photoionization of the solid matrix (by means of a medium-pressure Hg lamp) produces one cation for every neutral that disappears¹¹, it is possible to find the molar absorptivity of the cation visible band(s). This in turn can be related to the absorptivity of the cation infrared bands by the slope of the correlation plot. Then the integrated absorption intensity is approximately given by the relation $I = 2.303 \epsilon_{\text{max}} \Delta\nu_{1/2}$, where ϵ_{max} is the molar absorptivity maximum and $\Delta\nu_{1/2}$ is the bandwidth (full width at half maximum).

The frequencies (and wavelengths) of the infrared bands of the four PAH cations studied so far are presented in Table 1. For these PAHs, the intensities of the infrared bands of the ions are significantly different from the band intensities of the neutrals. Figure 2 shows this for Py and Py^+ . In neutral Py, the most intense band is the out-of-plane wag at 843 cm^{-1} . In cationic Py,

the most intense bands are the C–C stretch and C–H in-plane bending modes in the $1,000\text{--}1,600\text{ cm}^{-1}$ region. No new bands were observed in the C–H stretch region. The integrated intensities of neutral and cationic Py, and of the other three PAHs and their cations, are given in Table 2 in the regions of astrophysical interest (11.3 , $7.8+8.6$, 6.2 and $3.3\mu\text{m}$). These regions were chosen under the supposition that the bands seen for the interstellar medium (ISM) result from a mixture of different PAHs; the identification and precise percentage of each is, of course, uncertain at present. In addition, in Table 2 the intensities for each infrared region are summed for the four PAHs and their cations. On ionization, the intensity in the $11.3\mu\text{m}$ region decreases by a factor of ~ 2 , whereas in the $7.8+8.6\mu\text{m}$ region it increases by a factor of 7.9 . In the $6.2\mu\text{m}$ region the intensity also increases, by a factor of 5.5 . (The change in the $3.3\mu\text{m}$ region is problematic as no new bands attributable to the cations could be discerned there.) It seems probable that as more and larger PAHs are investigated, these sums and their relative values will vary. But it also seems likely that the striking decrease in intensity in the C–H out-of-plane wagging region and increase in intensity in the in-plane C–C stretching and C–H bending regions will remain valid.

Two separate studies, one over 24 interstellar sources¹² and the other over 77 sources¹³, have recently given the intensity ratio $I(7.8+8.6\mu\text{m})/I(11.3\mu\text{m})$ as about 7 (6 and 8.3, respectively). This ratio for the neutral PAHs studied here is only 0.45, whereas for the ionized PAHs it is 6.6, a value in good agreement with the ISM value, considering the small sampling of PAHs probed here. Furthermore, the average ratio $I(7.8+8.6\mu\text{m})/I(3.3\mu\text{m})$ determined¹² from the 24 ISM sources is 53, with a range of 8 to 120. The four neutral PAHs yield a ratio of 0.57. The ratio for the ions cannot be determined from our data; however, if we assume that C–H stretches for the cations are only 10% the intensity of the neutral stretches (the order of magnitude calculated by *ab initio* theory for naphthalene¹⁴ and anthracene⁵), this ratio becomes ~ 46 , again in good agreement with the ISM value.

The variation in the ratio from source to source in the ISM could result from (1) variations in the telescopic aperture used to determine the emission from different sources in different wavelength ranges, (2) differences in the spectral energy distribution fields around the various sources, (3) a mixture of ionized PAHs (the 'PAH soup') near that source which differs from our mixture, or (4) variation of the percentage of ionized and neutral PAHs from source to source. Although our results do not bear on the first two reasons, they do affect the latter two. Our findings provide evidence that PAHs in the ISM cannot exist solely as neutral species, that in regions of the ISM where the ratio $I(7.8+8.6\mu\text{m})/I(11.3\mu\text{m}$ or $3.3\mu\text{m})$ is low, the PAHs exist both as ions and neutrals, and that in regions where the ratio is large, the PAHs are present primarily as cations. □

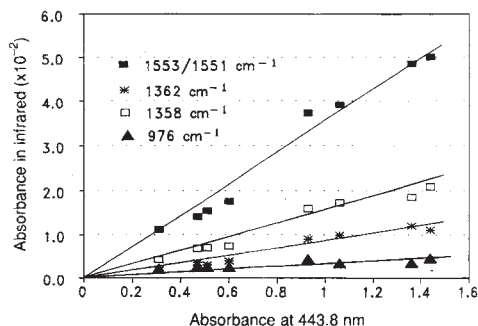


FIG. 3 Correlation plots of the 443.8-nm absorbance band of pyrene cation with four prominent infrared bands.

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Explosions of small Spacewatch objects in the Earth's atmosphere

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RECENT observations with the Spacewatch telescope indicate that the flux of Earth-crossing objects with diameters below about 50 m is some 10–100 times higher than predicted by simple extrapolation from the known main-belt asteroid population^{1,2}. This might seem to imply³ a significantly greater terrestrial hazard from atmospheric explosions such as those that occurred over Revelstoke or Tunguska^{4,5}. Here I show that explosions due to Spacewatch objects with diameters less than 50 m (having kinetic energies below about 10 megatonnes high-explosive equivalent) typically occur too high in the atmosphere to cause substantial surface damage. Exclusive of relatively rare iron objects, no comet or asteroid with an energy below ~2 megatonnes threatens the Earth's surface. The high flux of small Earth-crossing objects identified by Spacewatch therefore does not imply a greater terrestrial hazard.

Table 1a lists the 12 Spacewatch objects with diameters below ~50 m discovered so far (ref. 2 and D. Rabinowitz, personal communication). The semimajor axes (a), eccentricities (e), and inclinations (i) of these objects are also listed. From these orbital elements, I have calculated the probability and velocity of collision with Earth for each object, using Öpik's^{6,7} equations, modified⁸ to account for orbits that are not fully Earth-crossing. Table 1a also gives approximate diameters for each object, assuming² spheres of albedo 0.0945, the geometric mean between the albedos of S-type and C-type asteroids (0.186 and 0.048, respectively^{1,2}; these values are taken below to correspond to stony and carbonaceous compositions).

The Öpik equations used here^{7,8} incorporate the Earth's orbital eccentricity, but break down for Earth-crossing objects with extremely low inclinations⁷. The remarkable object 1991 VG has an inclination of only 0.25°, leading to the exceptionally high probability of impact with the Earth of 4,200 per thousand million years (Gyr^{-1}). (Impact probabilities vary as $(\sin i)^{-1}$; the mean probability⁹ for an Earth-crossing object to collide with the Earth is ~4 Gyr^{-1} .) The equations are valid provided⁷ the ratio of the Earth's gravitational capture radius $R_E [1 + (V_{\text{esc}}/V_{\infty})^2]^{1/2}$ to $a_E \sin i$ is $\ll 1$, where $R_E = 6,371$ km is the Earth's physical radius, $V_{\text{esc}} = 11.2$ km s⁻¹ Earth's escape velocity, a_E Earth's semimajor axis, and V_{∞} is the object's velocity at infinity. This ratio lies in the range 10^{-4} – 10^{-3} for all objects in Table 1a, except 1991 VG, for which it is 0.06.

1991 VG entirely dominates the characteristics of 'typical' terrestrial collisions by Spacewatch objects. For example, a 'typical' terrestrial collision velocity for these objects—weighted appropriately by collision probabilities—would simply be 11.4

km s⁻¹. It has been speculated, although not demonstrated, that 1991 VG is a human artefact². Excluding 1991 VG, the median, average and root-mean-square terrestrial impact velocities for the Spacewatch objects are 13.3, 14.3, and 14.4 km s⁻¹, respectively, compared with impact velocities for the previously known Earth-crossing asteroids of 15, 17 and 18 km s⁻¹ (ref. 10).

The mean impact probability for the objects in Table 1a (excluding 1991 VG) is 29 Gyr^{-1} , a factor of ~7 higher than that for the other Earth-crossing asteroids. Therefore the higher flux of near-Earth objects discovered by Spacewatch does not necessarily require a much higher population of these objects than previously estimated. The flux is enhanced simply because the objects are in unusually Earth-like orbits.

Rabinowitz's analytical estimate of cumulative impact rates¹, as a function of object mass, was made before the Earth-like nature of the Spacewatch objects' orbits was evident², and assumed a terrestrial collision velocity of 20.1 km s⁻¹. Beginning instead with the median velocity of 13.3 km s⁻¹ calculated above, these collision fluxes may be recalculated, then cast in terms of the frequency of impacts of a given energy. With these modifications, the analytical estimate yields one 680 kilotonne (3.2×10^7 kg) impact per 21 years, and^{3,4} 21-kton (1.0×10^6 kg) impacts per year. (Note, however, that Rabinowitz's preferred numerical flux estimates are a factor ~2 higher than his analytical estimates at these masses.) These results may be

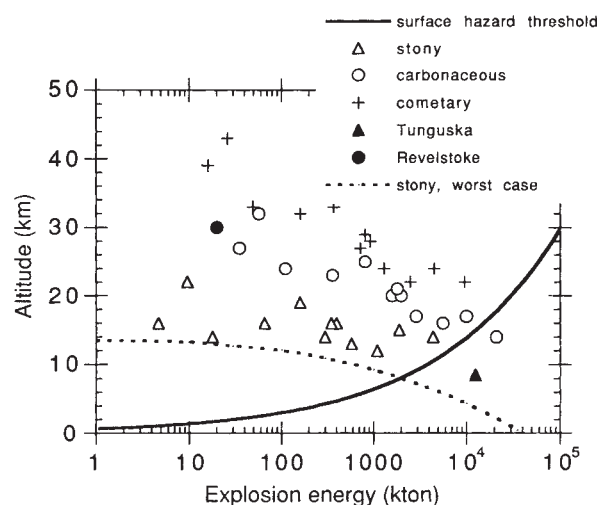


FIG. 1 Explosion altitude in the terrestrial atmosphere as a function of energy, for small Spacewatch asteroids. The Tunguska and Revelstoke explosions are plotted for comparison. Explosions occurring above the solid 'surface hazard threshold' line do not pose a significant threat to the Earth's surface. Exclusive of iron objects, no asteroid or comet of a given energy will explode below the 'worst case' line, which indicates the deepest possible atmospheric penetration for a stony asteroid of a given energy.

TABLE 1 Details of small Spacewatch objects

1a, Impact velocities and probabilities

Object	<i>a</i> (AU)	<i>e</i>	<i>i</i> (deg)	Diameter* (m)	Impact velocity (km s ⁻¹)	Impact probability (Gyr ⁻¹)
1991 BA	2.24	0.68	1.96	7	21.2	7.1
1991 TT	1.19	0.16	14.8	28	13.9	49
1991 TU	1.42	0.33	7.68	8	13.7	11
1991 VA	1.43	0.35	6.52	17	13.9	11
1991 VG	1.05	0.08	0.25	13	11.4	4,210
1992 DU	1.16	0.18	25.1	44	17.8	7.2
1992 JD	1.03	0.03	13.6	44	13.3	115
1992 YD3	1.17	0.14	27.8	55	18.6	6.8
1993 BD3	1.62	0.37	0.9	28	15.7	59
1993 DA	0.94	0.09	12	36	12.9	46
1993 FA1	1.42	0.29	21	26	16.5	2.6
1993 HP1	1.97	0.61	7.9	18	19.3	2.4

b, Airburst energy and altitude

Object	Stony asteroids			Carbonaceous asteroids			Comets†	
	Radius‡ (m)	Energy (kton)	Altitude (km)	Radius (m)	Energy (kton)	Altitude (km)	Energy (kton)	Altitude (km)
1991 BA	2.5	9.6	22	4.9	57	32	26	43
1991 TT	10	300	14	20	1,600	20	720	27
1991 TU	2.9	4.7	16	5.6	35	27	16	39
1991 VA	6.1	66	16	12	360	23	160	32
1991 VG	4.6	18	14	9.1	110	24	49	33
1992 DU	16	1,900	15	31	10,000	17	4,500	24
1992 JD	16	1,100	12	31	5,600	16	2,500	22
1992 YD3	20	4,400	14	39	21,000	14	9,600	22
1993 BD3	10	400	16	20	2,000	20	920	28
1993 DA	13	580	13	25	2,900	17	1,300	24
1993 FA1	9.3	350	16	18	1,800	21	810	29
1993 HP1	6.4	160	19	13	810	25	370	33

*These diameters assume² an albedo of 0.0945, the geometric mean between a spherical S-type asteroid and a C-type asteroid, with albedos of 0.186 and 0.048, respectively.

†Comet radii are taken to equal those of C-type asteroids (albedos assumed equal).

‡These are radii for spherical objects. The airburst model used here⁴ treats objects as initially 'cubical' cylinders; I take these objects to have masses (hence, explosion energies) identical to those listed (and therefore slightly different cylindrical radii). Radii and explosion energies are given here to two significant figures for the different models considered; however, fundamental uncertainties (such as whether the object is S-type or C-type) guarantee an overall uncertainty of a factor of at least ~2 in radius, and ~2 in energy.

compared to the Spaceguard Survey's estimate⁵ of one 20-kton impact per year.

The latter estimate is derived from the lunar cratering record¹¹, which shows an excess of craters at sizes corresponding to objects in the size range where Spacewatch finds an enhanced flux. But this flux was found¹ to be higher than implied by the lunar extrapolation. With the lower impact velocities determined here, mass estimates from lunar cratering increase by a factor of ~1.6, bringing the Spacewatch and lunar flux estimates into closer agreement.

The Spacewatch results seem to suggest a considerable terrestrial threat due to sub-megatonne impacts. On the other hand, they must be reconciled with the apparent fact that human civilization does not suffer frequent surface explosions in the 10–1,000-kton range. If most Spacewatch objects were small comets¹, they might dissipate their kinetic energy too high in the atmosphere to cause surface damage³. I demonstrate here, however, that this conclusion holds if the Spacewatch objects are stony or carbonaceous asteroids as well, as these objects will catastrophically disrupt too high in the atmosphere to cause

ground damage. Using a code successfully employed to model the Tunguska and Revelstoke atmospheric explosions⁴, I have simulated the entry of the Spacewatch objects into Earth's atmosphere, assuming iron, stony, carbonaceous and cometary compositions. All objects are taken to be incident on the atmosphere at the most probable impact angle of 45°. The results of these simulations are given in Table 1b. The altitude at which an object is taken to explode is that at which, once disrupted, it deposits maximum kinetic energy per unit distance⁴. The explosion energy is taken to be that deposited by the object after it has flattened out to twice its initial radius, typically within 10% of its incident kinetic energy. Iron objects, discussed further below, typically crater the terrestrial surface.

The albedos of the Spacewatch objects are not known. Table 1b derives object radii based on the choices^{1,2} of S- and C-type albedos. Comets are assigned C-type albedos. Following ref. 4, densities are set to 3.5, 2.2 and 1.0 g cm⁻³, and material strengths are taken to be 10⁸, 10⁷ and 10⁶ dyn cm⁻², for stony, carbonaceous and cometary objects respectively. These choices

of albedo (and hence radii) lead to the C-type and cometary candidates being more energetic than their stony counterparts.

Figure 1 plots explosion energy against altitude for the 12 objects in Table 1b. Possible stony, carbonaceous and cometary compositions are indicated. The Tunguska and Revelstoke explosions are shown for comparison. In addition, two important thresholds for surface destruction are shown.

Hills and Goda¹² present a formula for the radius r_4 at the ground below an explosion of energy E at altitude h out to which the resulting overpressure will exceed 4 p.s.i. (2.8×10^5 dyn cm⁻²). This is the overpressure at which trees will be felled and substantial damage to buildings will occur¹². Wood frame houses are destroyed at overpressures of 5 p.s.i. (ref. 13). Lower overpressures may also cause some damage; overpressures of 0.5–1 p.s.i. will break glass windows¹³, and an overpressure of 2 p.s.i. is the threshold at which these fragments will begin to be propelled with sufficient velocity to cause serious injury¹⁴. Nevertheless, 4 p.s.i. seems an appropriate choice as a definition of a 'substantial' surface hazard posed by an air explosion.

Citing an unpublished analysis of atmospheric nuclear tests by J. Solem, Hills and Goda¹² give $r_4 = 2.09 h - 0.449 h^2 E^{-1/3} + 5.08 E^{1/3}$, with E in megatonnes explosive equivalent and h in kilometres. This equation consistently relates treefall observations at the Tunguska site with microbarograph and other evidence (ref. 4 and refs therein) for a 10–20-Mton explosion at 8–9 km altitude, provided r_4 refers to the central circular treefall zone, excluding the lobes of the observed 'butterfly' pattern (perhaps caused by the ballistic wave from the terminal motion of the Tunguska object, before it exploded¹⁵). The altitude h above which an airburst does not threaten the surface is given by setting $r_4 = 0$, or $h = 6.42 E^{1/3}$, shown as the solid curve in Fig. 1. Airbursts occurring above this line pose no substantial threat to the ground.

The above equations were derived for nuclear weapons, where ~50% of the explosion energy is lost to thermal radiation or other energy sinks apart from shock production. Lower-temperature bolide explosions may produce shock waves more efficiently; one simulation¹⁶ finds only 12–25% of bolide explosion energy lost to radiation. As energy enters the above expression only to the 1/3 power, however, the resulting uncertainty is small.

Figure 1 shows that Spacewatch objects with energies below ~10 Mton (at 13.3 km s⁻¹, a stony asteroid ~32 m in radius) do not threaten the surface. Carbonaceous and cometary objects of a given energy always pose less of a threat than stony ones, as they explode higher in the atmosphere because of their lower strengths and densities. What is the lowest energy stony object that can threaten the surface? For a given energy, deepest penetration into the atmosphere occurs for the lowest possible

velocity (as catastrophic disruption begins at a pressure proportional to ρv^2 , where ρ is atmospheric pressure and v is bolide velocity), or 11.2 km s⁻¹ on Earth, and for normal incidence. The dotted line in Fig. 1 shows altitude as a function of energy for a series of such 'worst-case' stony asteroid explosions in the terrestrial atmosphere. No stony (and of course no carbonaceous or cometary) asteroid with kinetic energy below ~2 Mton will cause significant ground damage.

It is not known what fraction of the small Spacewatch objects are composed of iron. Iron–nickel meteorites constitute only ~8% of observed meteorite falls, and probably a much smaller fraction of the meteoritic bodies incident on the atmosphere¹⁷. However, iron objects make up about 6% of observed main-belt asteroids¹⁸, and about an equal fraction (though the statistics here are poor, and these percentages ignore selection effects) of Earth-crossing asteroids¹⁹.

Each of the Spacewatch objects in Table 1, if of iron composition, would crater the ground. Those with velocities above ~14 km s⁻¹ would experience ρv^2 pressures sufficient to cause disruption before impact, possibly yielding a crater-strewn field such as that left by the ~10-kton Sikhote-Alin explosion in 1947 (ref. 20). If 6% of the Spacewatch objects were iron, globally there would be one ~20-kton explosion every ~5 years, and one ~700-kton event every ~350 years. If the former are really occurring, they clearly pose no hazard, as nearly all evidently pass unnoticed. The latter would excavate one ~500-m crater on land every 1,400 years, and the ocean impacts would not cause appreciable tsunamis¹²; again this threat seems small.

On the other hand, if Spacewatch objects originate as, for example, lunar impact ejecta (possibly accounting for their Earth-like orbits), iron objects would be entirely absent. Indeed, limited spectral observations suggest that Spacewatch asteroids are distinct from known asteroid types². It would be of considerable interest to make spectral comparisons of Spacewatch objects with lunar and martian meteorites.

The Spacewatch discovery^{1,2} of a higher flux of small Earth-crossing objects does not imply a significantly higher terrestrial impact hazard, despite the prediction of several or more 20-kton impacts every year. But even if these explosions are not felt at the ground, it is at first startling that they could be occurring so frequently in Earth's atmosphere virtually unnoticed. A number of United States (and, presumably, former Soviet) defence satellites are capable of detecting atmospheric events above a certain luminosity threshold, although most such detections are discarded as irrelevant for defence purposes. The Spacewatch results lend urgency to the declassification of the relevant defence data, as well as to efforts to improve the reporting performance of existing satellites²¹. □

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Evidence for a near-Earth asteroid belt

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IN January 1991, the 0.9-m Spacewatch telescope made the first observation¹ of an asteroid outside Earth's atmosphere but in the neighbourhood of the Earth-Moon system. Since then, more than 40 Earth-approaching asteroids (defined as objects with perihelia of less than 1.3 AU) have been discovered, including 13 smaller than 50 m. Using these data, one of us (D.L.R.) has shown² that there is an excess of Earth-approaching asteroids with diameters less than 50 m, relative to the population inferred from the distribution of larger objects. Here we argue that these smaller objects — characterized by low eccentricities, widely ranging inclinations and unusual spectral properties — form a previously undetected asteroid belt concentrated near Earth. The recent discovery of additional small Earth-approaching asteroids supports this conclusion.

Table 1 lists the semimajor axis (a), eccentricity (e), inclination (i), perihelion (q), aphelion (Q), absolute magnitude (H), and diameter (d) for all of the small asteroids with $H > 25.0$ ($d \leq 50$ m) that have been detected with the Spacewatch telescope (we will refer to these as the small Earth-approachers, or SEAs). Values for d are the geometric mean between bright and dark estimates assuming spherical shapes with high and low albedos (0.186 and 0.048, respectively)². All but three have q or Q between 0.92 and 1.02 astronomical units (AU) and nearly half have $e < 0.2$. In ref. 2 the clustering of q values near 1.0 AU was noted, but there were not enough observations to rule out observational bias as the cause.

A bias exists because the volume of space that is searched with a telescope is finite in extent. Those objects with orbits that bring them most often into the search volume, and keep them there the longest, dominate the observed population. Because the extent of the search volume depends on d (the largest objects can be seen farthest away), bias is also size-dependent. For the small asteroids listed in Table 1, the limiting distance for

discovery ranged from 0.005 to 0.05 AU, and the bias towards objects with $q \approx 1.0$ AU and $e \approx 0$ is strong. On the other hand, there is also a bias against faint objects with Earth-like orbits because most of these are detected by visual recognition of their trailed images. As discussed in ref. 2, these faintest objects cannot be detected when their apparent rate of angular motion is less than 8° per day. With the recent discovery of four new asteroids, each with $e < 0.2$, we can now discriminate the observed cluster of Earth-like orbits from these effects of observational bias.

Figure 1 shows aphelion against perihelion for a fictitious population of asteroids (small triangles) with $25.0 < H < 28.0$ ($10 \text{ m} \leq d \leq 50 \text{ m}$) that would be observed with the Spacewatch telescope if they had an orbit distribution similar to the known Earth approachers, most of which are larger than 100 m in diameter. Also shown are the actual discoveries from Table 1 in the same magnitude range (large triangles). We aim to show that the distribution that we observe for SEAs is significantly different from the orbital distribution of known Earth approachers which are larger than 100 m.

The simulated observations plotted in Fig. 1 show the orbital distribution of the known population taking into account the effects of observational bias. These are the orbits that would be preferentially observed given the constraint that they must be observed very near Earth. They are the output of a computer program, described in ref. 2, which we have used to model the bias of the Spacewatch search. This program generates a large ensemble of fictitious asteroids with orbital elements and absolute magnitudes distributed according to parameters set by the programmer. It then assigns to each object in the ensemble a mean anomaly chosen at random from the range 0 to 2π . The simulated points in Fig. 1 are the orbits of those objects in the simulated ensemble that appear within the detection volume of the telescope, defined by the limiting magnitude and limiting angular rate for recognition, and by the position and extent of the searched sky area. Objects with preferentially observable orbits dominate the simulated distribution.

We exclude our observations of 1992 YD₃ and subsequent discoveries from Fig. 1 and consider their implication separately, below, because they were detected after a recent improvement to the Spacewatch telescope not accounted for in this calculation. We also exclude our observations of asteroids with $H > 28$ because too much computer time would have been required to extend the simulation to objects discovered as near to Earth as these.

Details of the simulation are given in ref. 2. Briefly, the orbits

TABLE 1 Orbit, brightness and size for small Earth approachers

Name	Discovery date (UT)	a (AU)	e	i (°)	H	d (m)	q (AU)	Q (AU)
1991 BA	91 Jan 18	2.243	0.682	1.96	28.9	7	0.72	3.77
1991 TT	91 Oct 6	1.193	0.161	14.76	26.0	28	1.00	1.39
1991 TU	91 Oct 7	1.416	0.333	7.68	28.5	8	0.94	1.89
1991 VA	91 Nov 1	1.429	0.352	6.52	27.0	17	0.93	1.93
1991 VG	91 Nov 6	1.047	0.076	0.25	27.6	13	0.97	1.13
1992 DU	92 Feb 26	1.160	0.175	25.06	25.0	44	0.96	1.36
1992 JD	92 May 3	1.034	0.032	13.59	25.0	44	1.00	1.07
1992 YD ₃	92 Dec 27	1.169	0.139	27.75	25.5	55	1.01	1.33
1993 BD ₃	93 Jan 26	1.618	0.369	0.87	26.0	28	1.02	2.22
1993 DA	93 Feb 17	0.936	0.093	12.33	26.6	21	0.85	1.02
1993 FA ₁	93 Mar 28	1.432	0.292	20.57	26.0	28	1.01	1.85
1993 HD	93 Apr 20	1.445	0.664	5.74	25.0	44	0.49	2.40
1993 HP ₁	93 Apr 27	1.921	0.493	7.78	27.0	17	0.97	2.87
1993 KA	93 May 17	1.257	0.199	6.10	26.0	28	1.01	1.51
1993 KA ₂	93 May 21	2.227	0.775	3.18	29.2	6	0.50	3.95

Uncertainties are roughly $\pm 1\%$ for semi-major axis, a , eccentricity, e , inclination, i , perihelion, q , and aphelion, Q ; ± 0.5 for absolute magnitude, H ; and a factor of 2 for diameter, d .

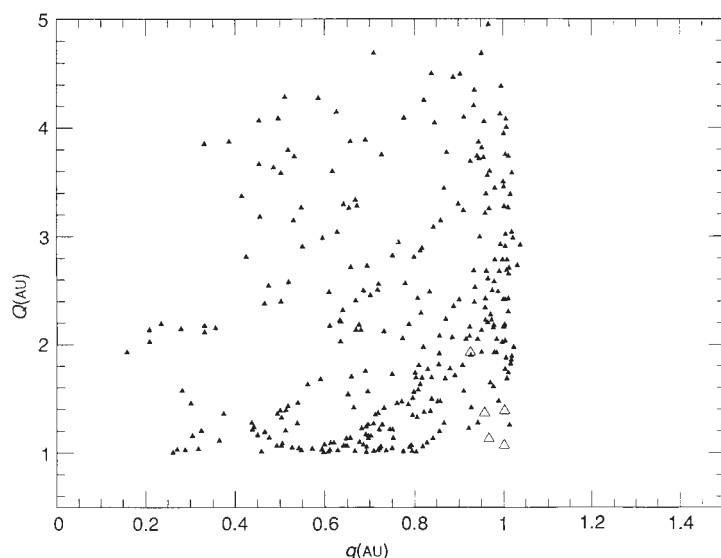


FIG. 1 Aphelion (Q) against perihelion (q) for simulated (small triangles) and observed (large triangles) Earth approachers in the size range 5 to 50 m ($28.0 > H > 25.0$).

of the known population of Earth approachers larger than ~ 100 m (as of 1993 January 15) were first debiased by modelling the searches made at Palomar with the 0.46-m Schmidt telescope. This is the telescope that has discovered most of the known Earth approachers³. Starting with this debiased distribution of orbits, the program was used a second time to model the bias of the Spacewatch search shown by Fig. 1. The absolute magnitudes for the simulated ensemble were distributed so that the fraction with magnitude less than H was proportional to $e^{1.8H}$. This is the steepest magnitude-frequency relation consistent with the observed distribution², and therefore introduces the strongest bias towards Earth-like orbits.

The existence of a distinct population with Earth-like orbits is suggested by the comparison of simulated and observed distributions shown in Fig. 1. Four of the five points in the observed distribution fall in the range $0.9 \text{ AU} < q < 1.1 \text{ AU}$, $1.0 \text{ AU} < Q < 1.4 \text{ AU}$, where only three of the 337 simulated orbits occur. If the simulation is realistic, then the probability of finding the five asteroids with their observed orbits is the same as the probability of randomly choosing five integers in the range 1

to (337/3) and obtaining at least four with the same value. That probability is 3.12×10^{-8} . It is more likely that the simulation is not a realistic representation of the distribution of orbits for Earth approachers in the 10–50 m range. In particular, there is a larger fraction of objects with both q and Q near 1.0 AU among these smaller objects than is present in the population of larger asteroids. 1992 YD₃, 1993 DA, and 1993 KA add weight to this conclusion because their orbits are Earth-like. However, their significance is less than for the other observations because they would not have been discovered without the recent telescope improvement which has biased the Spacewatch search more strongly towards objects in this region of orbital parameter space.

Figure 2a and b show the inclination distributions for the simulated and observed asteroid orbits from Fig. 1 with $q > 0.9$ AU, which covers the observed q range. Again, the observations do not match the prediction. Given that three of the five observations have $i > 10^\circ$ whereas only 19 of the 60 simulated orbits with $q > 0.9$ AU have $i > 10^\circ$, the probability for the observed ratio is 0.21 (the probability of randomly choosing five integers in the range 1 to (60/19) and obtaining at least three with the same value). Hence, it is likely that inclinations are more broadly distributed for the SEAs than for larger Earth approachers. Again, this conclusion is strengthened when the high inclinations for 1992 YD₃ and 1993 DA are considered ($i = 27.8^\circ$ and 12.3°). Because the improved search has increased the bias towards smaller inclinations, these observations should be given extra weight.

The unusual colours seen for some of the SEAs are shown in Fig. 3a and b, where magnitude differences through standard Johnson (B, V) and Cousins (R, I) filters are plotted. These measurements were made with CCDs (charge-coupled devices) on the 2.1-m telescope of the Kitt Peak National Observatory (1991 VA), the 2.3-m (1991 VG, 1992 JD) and the 1.54-m (1992 DU) telescopes of the University of Arizona. Also plotted are magnitude differences for large, main-belt asteroids ($d \approx 10$ km) observed during the Eight Color Asteroid Survey (ECAS)⁴. Transformations from ECAS filters (b, v, w, x) to B, V, R and I were determined from published observations of standard stars in both colour systems^{5–8}. The colours for three of the four asteroids (1992 DU, 1992 JD and 1991 VA) deviate from ECAS distribution by amounts greater than their measurement uncertainty. As the chance likelihood for these deviations is only 9%, we can conclude that the SEAs have unusual colours.

For 1991 VA, $Q = 1.93$ AU, outside the main SEA range. Given its unusual colour, small size and perihelion near 1.0 AU, however, this may be part of a broader population of objects associated with the SEAs. 1991 VG has been suspected to be

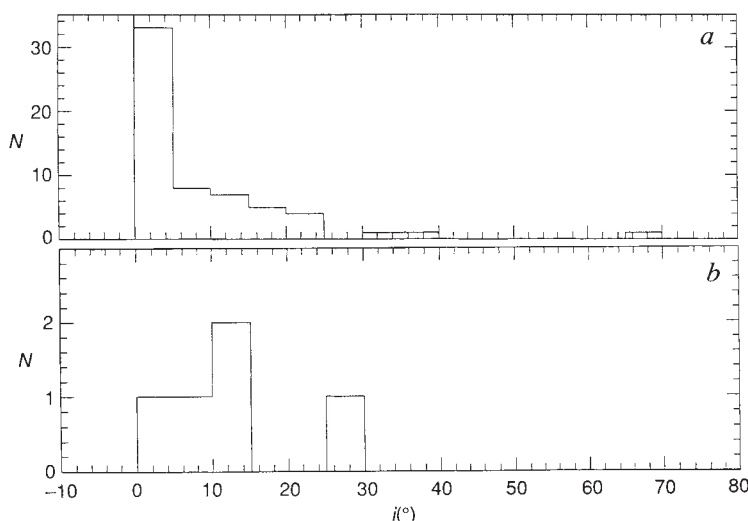


FIG. 2 Inclination distributions for the simulated (a) and observed orbits plotted in Fig. 1.

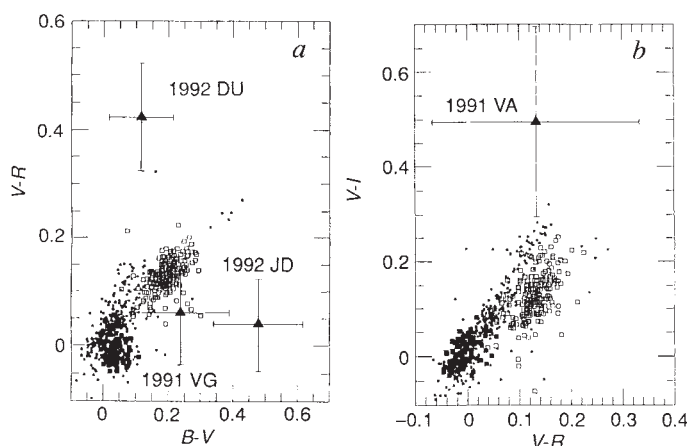


FIG. 3 Magnitude differences through Johnson (B, V) and Cousins (R, I) filters (4,400, 5,500, 6,700 and 8,100 Å, respectively) for small Earth approachers (triangles) and large, main-belt asteroids (solid boxes are spectral type C, open boxes are type S, star-shaped symbols are other types).

of artificial origin because of its extremely Earth-like orbit and unconfirmed evidence for rapid changes in its apparent magnitude, characteristic of artificial satellites^{9,10}. Excluding 1991 VG affects none of the above conclusions.

Where does this population of SEAs come from? In ref. 2 it was suggested that they are transported from the main asteroid belt to Earth-approaching orbits by resonant interaction with

Jupiter. Owing to their short, collisional lifetimes and enhanced production rate by collisional injection into resonant orbits, an excess would be created with $q \approx 1.0$ AU. It was also suggested that they might be the debris from extinct, short-period comets. However, neither of these explanations accounts for the small eccentricities of the SEAs. New sources may be required, such as lunar ejecta or an undetected population of asteroids at Lagrangian points on the Earth's orbit (analogous to the Trojan groups on Jupiter's orbit).

Further discoveries with the Spacewatch telescope of Earth approachers less than 50 m in diameter are consistent with the distribution of orbits reported here: most have perihelia near 1.0 AU, and many have low eccentricity. As we detect more SEAs with the Spacewatch telescope and observe them photometrically, we will obtain a clearer picture of their orbital distribution and physical properties. This will lead the way to an understanding of their origin. □

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One-dimensional reactivity in catalysis studied with the scanning tunnelling microscope

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THE scanning tunnelling microscope (STM) has yielded great insight into the structure of surfaces and into the dynamics of surface reconstruction and adsorption¹. We show here that it can also provide direct information about the microscopic mechanisms of catalytic reactions on surfaces. We have studied the oxidation of carbon monoxide on an oxygen-precovered rhodium (110) surface, a process related to the catalytic removal of CO in exhaust gases^{2,3}. The STM images show that the reactivity is strongly influenced by the oxygen-induced reconstructions of the surface. The reaction is initiated at high-energy adsorption sites, mainly at steps and domain boundaries of the adsorbed oxygen layer. The CO strips away one-dimensional islands of oxygen atoms on the reconstructed surface, proceeding in the [011] direction. More generally, these results show how the STM can provide insights into the microkinetics of surface reactions.

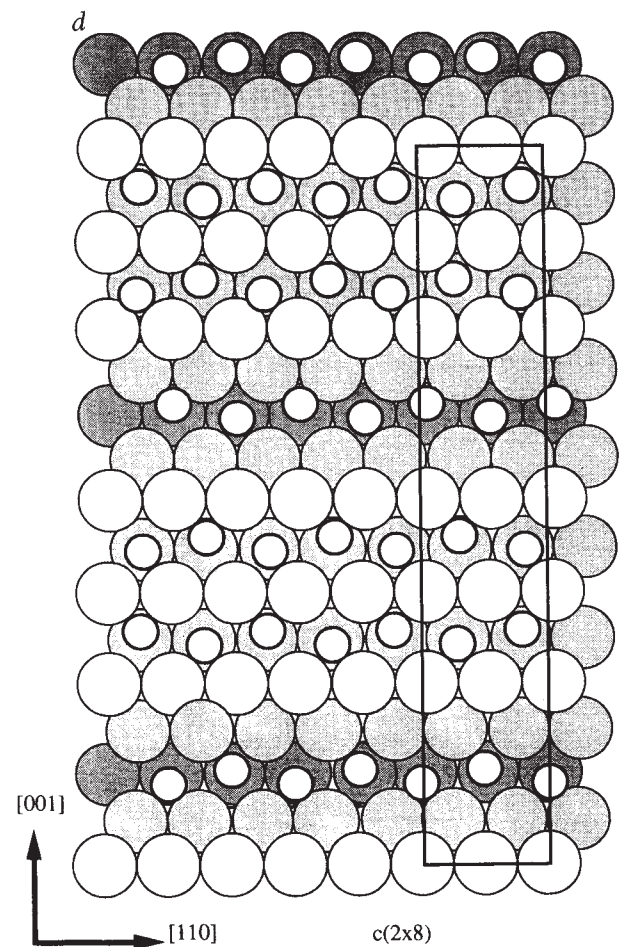
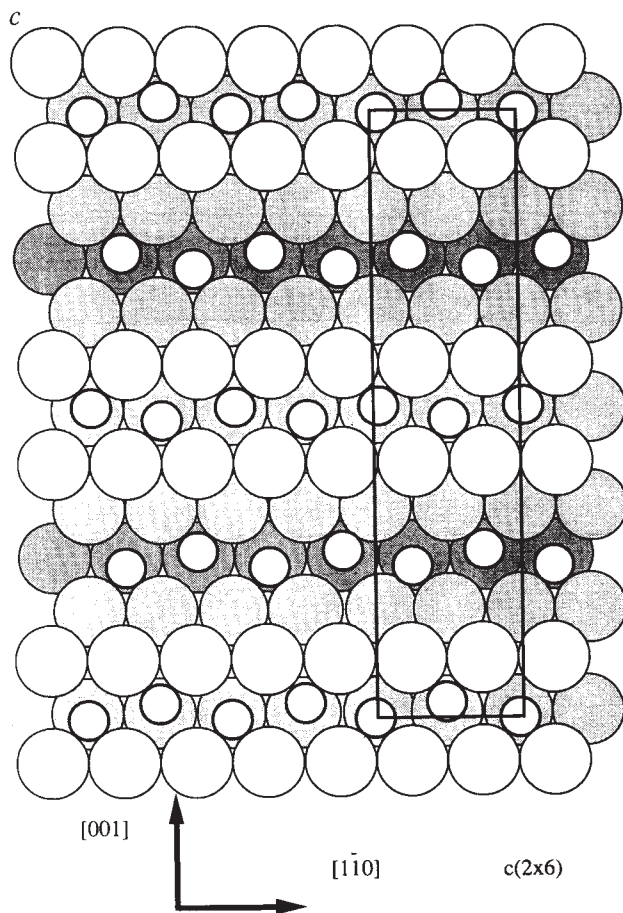
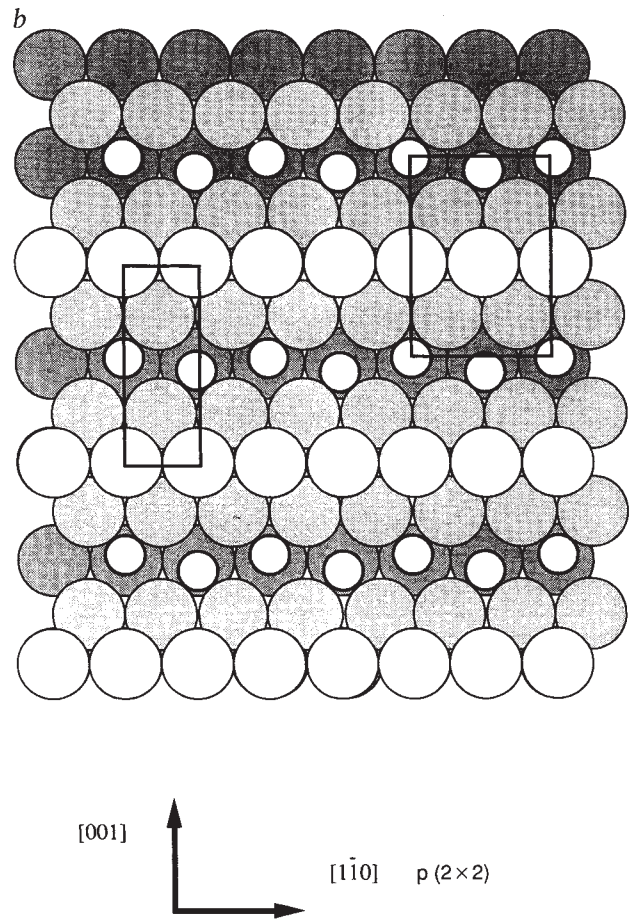
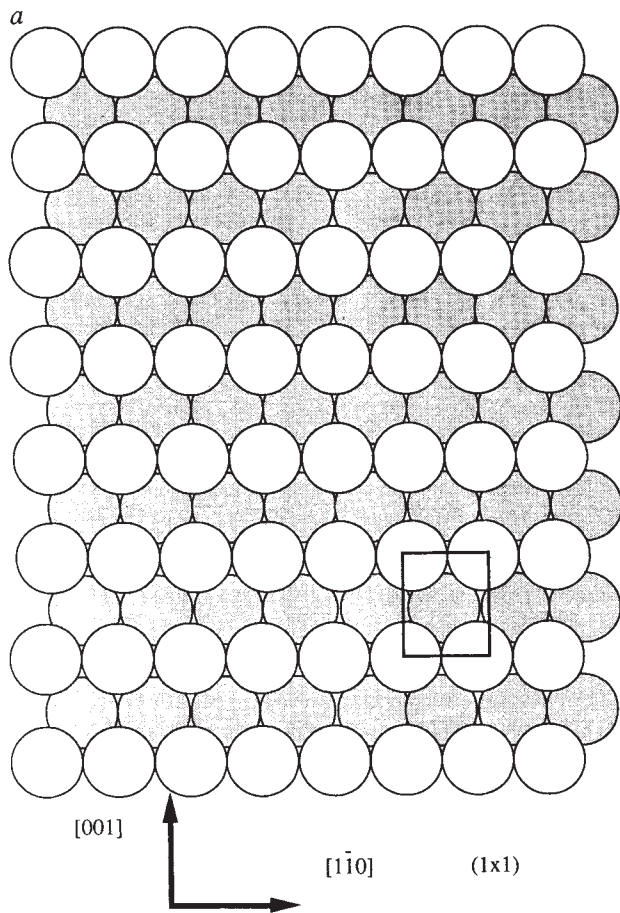
It is not uncommon for adsorbates to induce movement and reordering of the top layer of metal atoms, and indeed, it generally occurs for oxygen adsorption¹, as this initiates bulk oxidation. A range of other adsorbates also act this way,

including C (ref. 4), CO (refs 5, 6) and even hydrogen atoms^{7,8}. Gross reconstructions certainly occur when oxygen is adsorbed on Rh(110) as reported earlier^{9,10} (see Fig. 1). Our present experiments were done in a commercial ultra-high-vacuum chamber using an Omicron STM, with the rhodium (110) sample being prepared by cycles of argon-ion bombardment and annealing to 650 K. After exposing the sample to 5 langmuirs of oxygen (1 langmuir = 1.32×10^{-6} mbar s) while the sample was maintained at 620 K, a $c(2 \times 6)$ oxygen-induced low-energy electron diffraction pattern could be observed from this surface^{9–12}. A scanning tunnelling microscopy image of a typical area of the surface is shown in Fig. 2. Most areas show the $c(2 \times 6)$ oxygen-induced reconstruction, but there are several other types of structure present, including a fairly high density of step edges, terraces, domain boundaries, areas of higher oxygen coverage ($c(2 \times 8)$), and regions of a (1×2) -type reconstruction.

In Fig. 3, we show more detailed images of these structures. Figure 3a shows an image with both the $c(2 \times 6)$ and $c(2 \times 8)$: these reconstructions^{10,12} are modelled by one-dimensional islands of rhodium atoms with adsorbed oxygen atoms interspersed, together with periodic missing rows of Rh atoms in the [011] direction (Fig. 1). Figure 3b is an image of the $c(2 \times 8)$ and (1×2) -type reconstructions. At low oxygen coverages a (2×2) reconstruction is formed and a proposed model involves sub-

FIG. 1 Schematic plans of the proposed Rh structures involved in this work^{10,12}. a, (1×1) clean Rh(110); b, $p(2 \times 2)$ oxygen-induced structure (oxygen atoms are the unfilled circles) showing also the $p(1 \times 2)$ arrangement of surface Rh atoms; c, $c(2 \times 6)$; d, $c(2 \times 8)$. In all the oxygen-induced structures the top Rh layer has missing rows of Rh atoms in the close-packed direction, compared to the bulk crystal. It is possible that in some structures there is also a zig-zag reconstruction of the Rh atoms with alternate and opposite displacements in the [001] direction. The 3-fold site for oxygen adsorption is indicated by electron energy loss studies^{19,20}.

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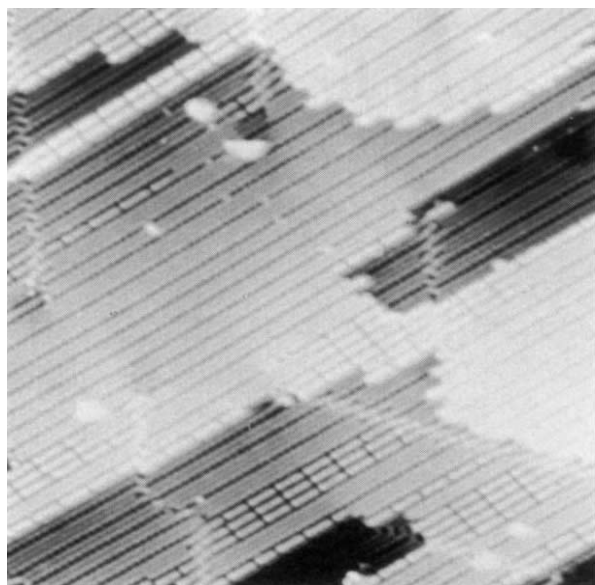


FIG. 2 400 Å × 400 Å scanning tunnelling microscopy image of the oxygen precovered (110) face of rhodium. The oxygen dose used in this experiment mainly produced areas of the $c(2 \times 6)$ reconstruction; also present are areas of the $c(2 \times 8)$ and (1×2) reconstructions. Images were taken with a sample bias of -2.0 V and a tunnelling current of 1 nA. We found that images of the surfaces did not vary significantly with the choice of tunnelling parameters used.

surface oxygen atoms arranged in a (2×2) pattern with top layer rhodium atoms in a (1×2) array¹². We believe, therefore, that the (1×2) areas that are observed here are actually areas of the (2×2) reconstruction, but that the oxygen atoms are not imaged. At higher oxygen coverages the $c(2 \times 6)$ reconstruction is seen, followed by the $c(2 \times 8)$ and then by the $c(2 \times 10)$ ^{9,11}. With the oxygen exposure used here, we have converted most of the surface to $c(2 \times 6)$ but there are also areas of higher coverage $c(2 \times 8)$ and lower coverage (1×2) . The proportions of the area with the $c(2 \times 6)$, $c(2 \times 8)$ and (1×2) reconstructions are roughly 48%, 35% and 17%, respectively. For many nonlocal probes the presence of multiple reconstructions, step edges and domain boundaries would be a disadvantage, but in scanning tunnelling microscopy, they provide an opportunity for simultaneous experiments on different features.

Carbon monoxide will react catalytically with oxygen on Rh(110), forming carbon dioxide which then desorbs leaving a

clean surface^{13,14}. Here, we dosed the surface with CO to see how this catalytic reaction proceeded on the different O-induced reconstructions. To do this, we raised the chamber pressure to 1×10^{-8} mbar of CO for ~ 7 minutes at a sample temperature of 300 K. Figure 4a shows an image of a typical area of the surface after exposure to CO. Bright regions have appeared on the surface. Examination of a higher-resolution image of one of these areas (Fig. 4b) shows that the bright regions are bounded by the missing rows and that areas of $c(2 \times 6)$ and $c(2 \times 8)$ coexist around them. This indicates that these lines appear bright not because of a tip effect but because of a real change on the sample. We believe that CO has removed the oxygen within these regions, so they represent either patches of clean surface or of surface with adsorbed CO. These patches are blurred, either because of the delocalized nature of the metal conduction-band electrons or because the CO is vibrating or translating during the time taken to image the surface. Our interpretation is supported by the results of Ertl¹⁵, who showed that the work function of the surface is lower for clean or CO-covered Pt(110) than for the oxygen-covered sample. In this case it would appear that the reaction proceeds linearly in the direction of the missing rows. The longest reacted line measured was 430 Å.

Examination of the width of the reacted region in Fig. 4b shows that it is part of a $c(2 \times 8)$ area. Within a fixed area (2×10^6 Å²) of the sample, we found that the ratio of reacted $c(2 \times 6)$ areas to reacted $c(2 \times 8)$ areas was 1 to 4.3. Weighting the reacted areas with the ratio of percentage areas converted to the two reconstructions, we find that the $c(2 \times 8)$ reconstruction is roughly 6 times more reactive to CO oxidation under the conditions of the experiment. The average lengths of reacted lines were 91 ± 43 Å and 89 ± 46 Å for the $c(2 \times 6)$ and $c(2 \times 8)$ respectively. This near-equal average reacted length suggests that once the reaction is initiated, it proceeds with similar velocity along areas of both reconstructions. We saw little evidence for reactions occurring on the (1×2) regions, in agreement with a previous Auger electron spectroscopy study¹².

The data also allow us to infer something about the initiation sites for the reaction. For both the clean and oxygen-reconstructed surfaces, step edges running parallel to the close-packed [011] direction are long and straight, whereas they are ragged in the [001] direction. Of 228 reacted lines examined, all but 37 had a boundary at a [001] step edge or domain wall. This suggests that these features serve as initiation sites for the oxidation process. Furthermore, sequential studies show the reaction front proceeding away from the step edge. It is likely that the steps represent the only free Rh sites available for the adsorption of CO which then enables the reaction to take place.

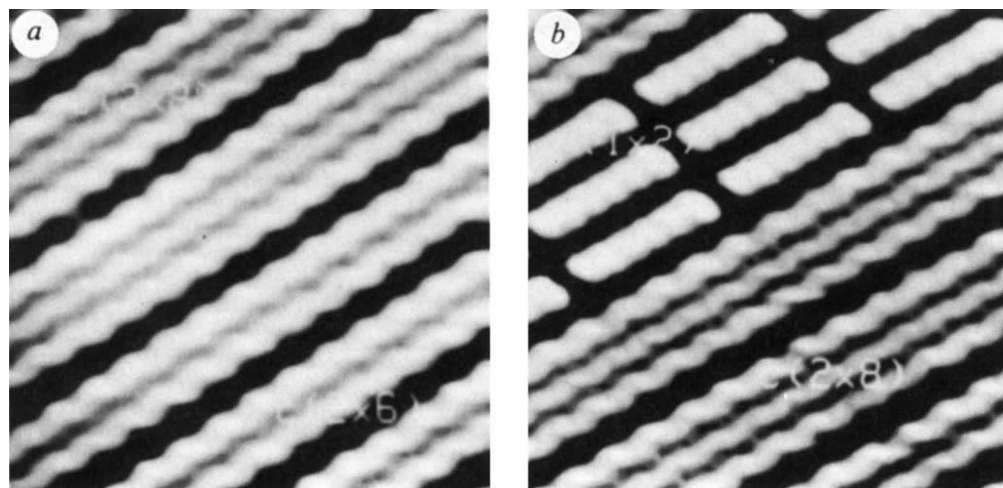


FIG. 3a, 60 Å × 60 Å image with the $c(2 \times 8)$ reconstruction present in the upper part of the image and the $c(2 \times 6)$ present in the lower part. b, 60 Å × 60 Å image showing both the (1×2) and $c(2 \times 8)$ reconstructions. The segmented nature of the (1×2) reconstruction is not understood.

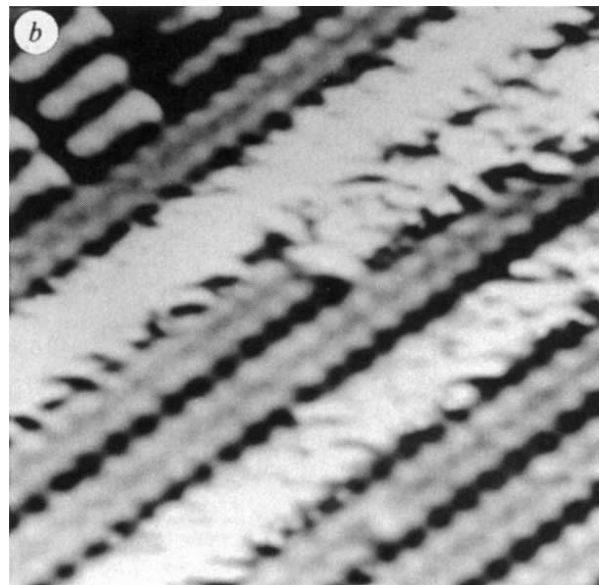
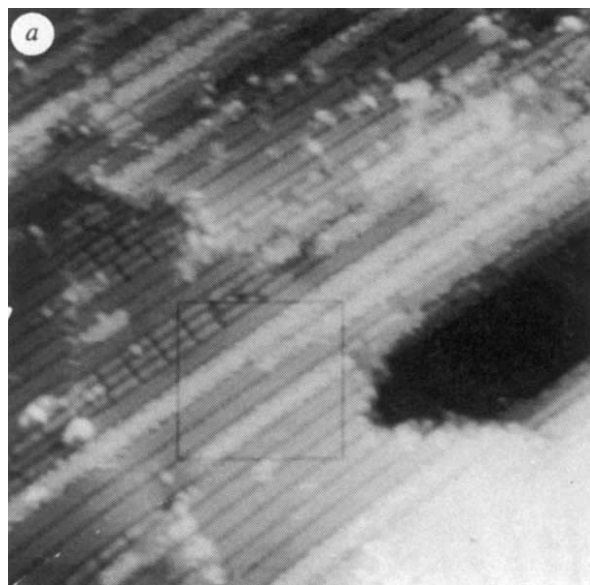


FIG. 4a, Typical $400 \text{ \AA} \times 400 \text{ \AA}$ image of an area of the surface following a 5 langmuir exposure to CO. The reaction appears to proceed in a highly directional manner on the surface. Reacted areas appear as bright, blurred lines. b, Expanded view of the area contained

within the box shown in a. This $105 \text{ \AA} \times 105 \text{ \AA}$ image shows reacted $c(2 \times 8)$ and $c(2 \times 6)$ areas coexisting with unreacted $c(2 \times 6)$ and (1×2) areas.

The reason that the step edges at the $c(2 \times 8)$ areas react fastest is probably a lower activation barrier to the O clean-off reaction¹³.

That the detailed nature of surface steps can play a critical role in catalytic reactions has been noted previously, for example in the hydrogenolysis of cyclohexane¹⁶. Although CO oxidation on rhodium seems to be structure-insensitive at low temperatures¹⁷, corresponding to high CO coverage, at the higher temperatures relevant to the situation in car exhausts the surface is dominated by oxygen atoms and the reaction is then sensitive to surface structure¹⁸. These and other studies have demonstrated the very high mobility of surface metal atoms, as manifested in a wide range of adsorbate-induced reconstructions, and it may be that this kind of diffusion creates the active sites during the catalytic process itself. Our results suggest that such processes should be accessible to direct study with the STM. □

Nitrogen transport by vertically migrating diatom mats in the North Pacific Ocean

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PHYTOPLANKTON production in the surface waters of stratified oceans is fed mainly by nitrogen that has been recycled within the euphotic zone¹. The nitrogen that is lost from surface waters as organic matter exported to the deep ocean must be balanced by inputs of new nitrogen to the upper ocean^{2,3}. Sediment trap studies² have shown that the $^{15}\text{N}/^{14}\text{N}$ ratio ($\delta^{15}\text{N}$) of the exported organic matter is higher than that of the suspended particulates, and suggest that the rich nitrate pool below the euphotic zone is the source of 'new' nitrogen for the upper ocean. Yet steep vertical concentration gradients suggest that diffusive upward transport of nitrate is extremely limited, raising the question of how the nitrate reaches the surface waters. Here we present evidence that abundant diatom (*Rhizosolenia*) mats migrate vertically between surface waters and deep nitrate pools in the central North Pacific Ocean. Rising mats contain significantly larger internal nitrate pools than sinking mats. Mat $\delta^{15}\text{N}$ is similar to that of the sub-nitricline nitrate, and consistently heavier than that of near-surface particulate organic matter. We conclude that *Rhizosolenia* mats may transport the equivalent of 50% of the new nitrogen requirements into the surface waters of the North Pacific gyre.

Rhizosolenia mats are buoyant, macroscopic (1–30 cm wide) diatom associations of multiple *Rhizosolenia* species occurring over broad expanses of the North Atlantic, North Pacific and Indian oceans^{4–6}. Mats are sporadic and occur at 10^{-3} m^{-3} or less

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in the North Atlantic⁵; no estimates are available for the Indian Ocean. In the central North Pacific gyre, however, mats are present almost all year and with abundances up to 4.9 m^{-3} (ref. 6). They are strongly buoyant (ascent rates of up to 6.4 m h^{-1}), are physiologically healthy despite low nutrient concentrations^{4,5}, and yield C-specific growth rates of 0.6 div day^{-1} (refs 4–6). Although they have been reported to contain N_2 fixing endosymbionts⁷, subsequent investigations have not confirmed this^{6,8}. Mats represented 98% of the biogenic silicate at a station north of Hawaii⁶.

Rhizosolenia mats were sampled by scuba divers along a transect of 31° N in September 1992 (Table 1). Mats were present at all stations at abundances of $>0.1 \text{ m}^{-3}$ with one surface maximum up to 6.0 m^{-3} (Fig 1). Suspension characteristics, a key aspect of vertical migration, varied considerably between stations with 0–80% of the mats sinking at any given station (Fig. 2). Afternoon-collected mats tended to sink more than morning mats, but we could not resolve spatial or temporal variability. Intracellular mat NO_3^- varied from 0.3 to 14.1 mM , with floating mats giving significantly higher average NO_3^- pools than sinking mats ($p = 0.015$, Table 1). *Rhizosolenia* mat $\delta^{15}\text{N}$ (range 3.3–5.2) averaged 4.4 ± 0.7 . Ambient suspended material had much lower $\delta^{15}\text{N}$ (–1.2 to 2.0) throughout the upper 100 m, suggesting a distinct N source (Fig. 3).

Diatoms do not accumulate internal NO_3^- pools when growing on ammonia^{9,10}. As the concentration of dissolved NO_3^- is $\leq 0.1 \mu\text{M}$ in the upper 100 m in this region¹¹, the presence of NO_3^- pools is considered key evidence for recent exposure to high NO_3^- (ref. 9). $\delta^{15}\text{N}$ serves as a natural tracer for N source, and permits identification of the mat N source. In the central Pacific gyre, sub-euphotic-zone NO_3^- $\delta^{15}\text{N}$ is relatively heavy (5–6)¹² whereas regenerated nitrogen $\delta^{15}\text{N}$ is substantially lighter¹³. Bulk phytoplankton in oligotrophic regions rely predominantly on regenerated N (ref. 14), thereby accounting for observations of ubiquitously low $\delta^{15}\text{N}$ (0–2) in euphotic-zone particulate organic nitrogen (PON)². As N utilization is complete in these waters, low $\delta^{15}\text{N}$ values cannot be attributed to isotopic fractionation during uptake and assimilation. The low $\delta^{15}\text{N}$ values for PON from our study sites are consistent with earlier observations (Fig. 2). Mats have substantially higher $\delta^{15}\text{N}$ (4.4) close to values for sub-euphotic-zone NO_3^- . These data show that NO_3^- is the mat's primary N source, in contrast to regenerated N for the bulk phytoplankton.

Contributions from regenerated N or N_2 -fixation would only lower $\delta^{15}\text{N}$ for the mats, making them more similar to PON in

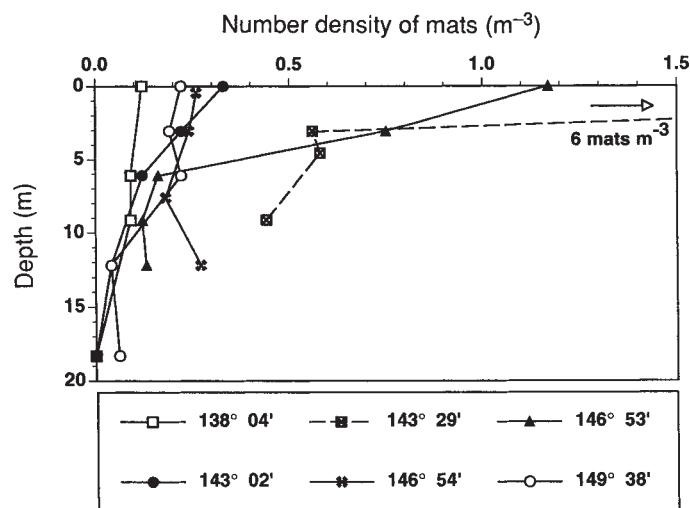


FIG. 1 Vertical distribution and abundance of *Rhizosolenia* mats. Mat abundance (average 0.5 m^{-3}) was measured by swimming a flowmeter-equipped 1 m^2 frame at defined depths and recording mats sampled⁶. Decompression limitations prevented our scuba divers going deeper than 20 m. Mats were visible to the limit of vertical visibility (30–40 m *in situ*).

isotopic composition¹⁵, as would isotopic fractionation in the uptake of NO_3^- (ref. 16). This effect seems to be minimal relative to the deep NO_3^- $\delta^{15}\text{N}$, and further indicates that once NO_3^- is taken up within the nitricline it is assimilated efficiently. Fractionation along biosynthetic pathways will lower cell $\delta^{15}\text{N}$ only if there is a considerable loss of intracellular intermediates.

The pronounced buoyancy changes and internal pool differences in rising and sinking mats suggests that N-stress is related to buoyancy, and these patterns are considered with a vertically migrating population acquiring NO_3^- at sub-nitricline depths. Together with the $\delta^{15}\text{N}$ evidence, the data indicate that migration is occurring. At present we cannot determine how frequently excursions occur.

Previous work on diatom ecology has focused on turbulence and nutrients as key determinants of suspension^{17,18}. The confirmation that diatom mats migrate vertically indicates that these concepts are inappropriate for explaining the occurrence and distribution of this large biogenic silicate pool. *Rhizosolenia*

TABLE 1 NO_3^- in ascending and descending diatom mats

Floating mats					Sinking mats				
Latitudes (N)		Longitudes (W)		Cell sap NO ₃ ⁻	Latitude		Longitude		Cell sap NO ₃ ⁻
31°	11.46'	143°	01.83'	7.5	31°	11.46'	143°	01.83'	1.1
31°	10.83'	143°	28.35'	9.9	31°	10.83'	143°	28.35'	0.3
31°	19.07'	153°	40.63'	14.1	31°	10.83'	143°	28.35'	4.6
31°	19.07'	153°	40.63'	6.1					
31°	19.07'	153°	40.63'	8.9					
31°	10.90'	156°	17.60'	11.6					
Average				9.7 ± 2.9	Average				2.0 ± 2.3

mats are clearly capable of extensive, directed movement within the epipelagic zone to exploit deep nutrient pools and surface irradiance, and probably use ionic regulation mechanisms similar to that used by *Pyrocystis* for buoyancy control^{6,20}. Mat vertical migration is similar to that of flagellated coastal dinoflagellates¹⁹ and the non-motile oceanic dinoflagellate *Pyrocystis*^{20,21}. Other oceanic genera such as the diatom *Ethmodiscus*²², solitary (non-mat-associated) *Rhizosolenia* and the prasinophyte *Halosphaera*²³ are positively buoyant, and are all character forms of oligotrophic oceans^{24,25}. These taxa are found primarily in stratified seas where light and nutrients are spatially separated, conditions known to favour vertically migrating taxa²⁶. These theoretical and behavioural data across diverse phylogenetic groups suggest that positive buoyancy is a general adaptation for depth-keeping and/or vertical migration in marine planktonic systems.

Phytoplankton vertical migration, unlike that by zooplankton²⁷, results in euphotic zone inputs of new nitrogen. Assuming 100% of mat N from NO_3^- , an average abundance of 0.5 mats m^{-3} over the upper 20 m, a mat C-specific growth rate of 0.6 div day^{-1} , and an average 7.4 $\mu\text{mol N per mat}^6$, we calculate a N input from migrating mats of 38.2 $\mu\text{mol N m}^{-2} \text{ day}^{-1}$. This is 2–27% of the estimated upward turbulent flux of NO_3^- (0.14–1.6 $\text{mM N m}^{-2} \text{ day}^{-1}$) for highly stratified systems in the North Atlantic^{3,28}. We chose to use Atlantic Ocean turbulent flux calculations because few estimates are available for the central Pacific gyre. The similarity in vertical density and nutrient structure suggests that the error in this estimate is small compared with the uncertainties of the biological component^{1,29}.

Physically driven, upward NO_3^- flux from the nitricline is consumed at the deep chlorophyll maximum well below the mixed layer and leads to low surface NO_3^- (ref. 30). Mat ascent to the mixed layer bypasses the sink and provides NO_3^- inputs to this region of extremely low f ratios (fraction of production supported by N remineralized in the euphotic zone^{31,32} otherwise dominated by herbivore-mediated ammonia regeneration¹⁴, with N_2 -fixation³³ and atmospheric inputs³⁴ as the only other new N sources. With a bulk carbon productivity of 0.5 $\text{mmol C m}^{-3} \text{ day}^{-1}$ in the upper 20 m (ref. 35). Redfield C/N ratios, and $f=0.05$, the estimated mat transport could represent 50% of the required new N inputs in the upper mixed layer.

We know of no data on mat remineralization rates, although both fish³⁶ and protozoans are reported to consume mats⁶. However, regardless of whether it is immediately exported or regenerated, carbon-fixation from vertically transported NO_3^- still represents new production relevant to carbon cycling. Grazing by herbivores and remineralization of mat nitrogen would lower the $\delta^{15}\text{N}$ (ref. 13), so the $\delta^{15}\text{N}$ difference between mats and suspended particulates does not preclude N flux

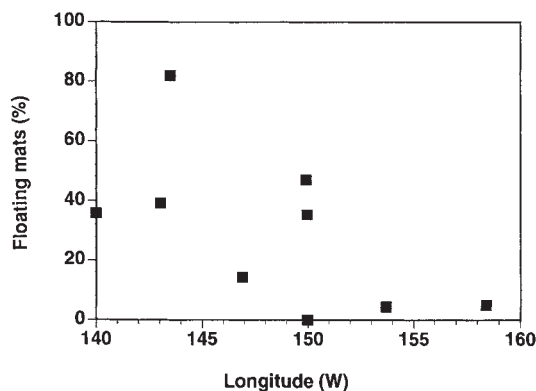


FIG. 2 Buoyancy characteristics along the transect at 31° N. See Table 1 for methodology.

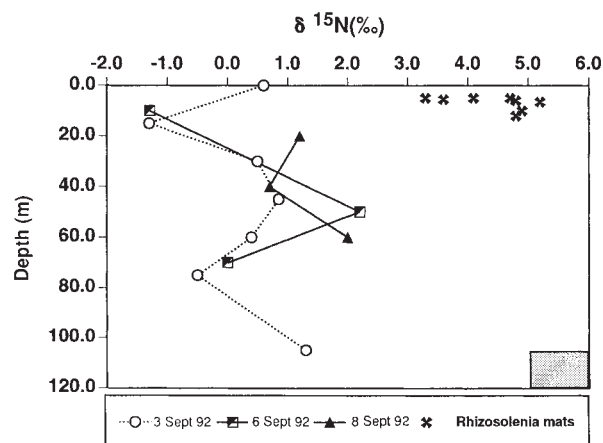


FIG. 3 $\delta^{15}\text{N}$ values of *Rhizosolenia* mats and suspended particulate material. Particulate profiles were collected at 19:30 3 September 1992, 19:00 6 September 1992, and 17:00 8 September 1992. Mats were collected between 09:00 and 16:00. Hatched area indicates NO_3^- $\delta^{15}\text{N}$ ratios below 200 m (ref. 12). Particulates were collected in 5-litre Niskin bottles, filtered onto precombusted quartz QMA filter; mats were filtered onto precombusted glass (GFF) filters. $\delta^{15}\text{N} = ((^{15}\text{N}/^{14}\text{N})_{\text{sample}} / (^{15}\text{N}/^{14}\text{N})_{\text{atmospheric}} - 1)$ and was analysed by mass spectrometry³⁷. Reproducibility = $\pm 0.15\text{‰}$. Mats were examined for zooplankton which are enriched in ^{15}N relative to phytoplankton (by about 3.5‰ per trophic level^{45,46}), and these were removed if present. As suspended particulate nitrogen is a mixture of phytoplankton, bacteria, microzooplankton and detritus, the actual difference between mats and other phytoplankton is larger than our observations indicate. High mat $\delta^{15}\text{N}$ supports observations that N_2 fixation is not occurring⁶.

between the two pools.

It follows that if mats are important in new N transport and new production in the North Pacific, they should directly or indirectly make large contributions to transport out of the euphotic zone. Floating sediment traps placed just below the euphotic zone in similar oligotrophic conditions in the North Atlantic show sinking particles to have values substantially higher than the bulk PON but similar to values for NO_3^- (ref. 2). Sargasso Sea $\delta^{15}\text{N}$ budgets also suggest fairly high exports of enriched ^{15}N out of the upper euphotic zone which must be balanced by new N inputs³⁷. There are no similar $\delta^{15}\text{N}$ data from the central Pacific gyre for comparison, but we expect fundamental similarities between these two highly stratified, oligotrophic regions.

We suggest that *Rhizosolenia* mat-mediated N transport between N-rich deep waters and N-poor surface waters is an important vector for vertical NO_3^- flux in the central Pacific gyre. The importance in other regions is clearly influenced by mat abundance. They are rare in the North Atlantic, but occur seasonally in the equatorial Atlantic³⁸. Little is known of other oceans. As noted above, other large buoyant phytoplankton, probably capable of vertical migration, are common in oligotrophic seas. In addition, potentially buoyant taxa can be an important component of sediment-trap collections³⁹. Vertical migration as a vegetative growth strategy or as a result of sexual reproduction⁴⁰ will clearly affect flux calculations, and these observations, model calculations⁴¹ and laboratory data on oceanic diatoms^{42,43} suggest that the emphasis on only the pico- or nanophytoplankton can overlook the role of algal behaviour in biogeochemical cycling. □

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Geochemical constraints on mantle melting during creation of the North Atlantic basin

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THE compositions of magmas produced by decompression melting of upwelling mantle rocks are sensitive to the extent and mean pressure of melting; these, in turn, depend respectively on the depth at which the solidus is encountered^{1,2} and on the thickness of the lithosphere, which provides a barrier to upwelling mantle^{2,3}. Here we report major- and trace-element data for lavas erupted during rifting of the Greenland–European continent ~60 Myr ago, which show a trend to higher extents of melting at lower pressures as rifting proceeded. We attribute these changes to progressive thinning of the continental lithosphere during the initial phase of magmatism. Our analysis also shows that mantle melting began well within the garnet stability field, supporting previous suggestions^{4–8} that anomalously hot mantle was present beneath the region at the time of rifting. The modest extents of melting that we infer for the earliest rift lavas can largely account for their high contents of incompatible elements, thus reducing the degree of geochemical enrichment ('plume-like' character) required in the mantle source region.

Igneous units up to 20 km thick have been inferred from marine seismic profiles along the Greenland and European margins^{9–11}. These regions of thick igneous crust roughly coincide with seafloor magnetic anomaly 24 (54 Myr; ref. 12) and overlap an abrupt transition from oceanic to continental structures¹³. Basaltic rocks from these units were retrieved during Ocean Drilling Program (ODP) leg 104 and Deep Sea Drilling Program (DSDP) leg 38 on Vøring Plateau, and from DSDP leg 81 on Rockall Plateau (Fig. 1).

Lavas landward of these submarine sections are sampled on the Faeroe Islands, the northern British Isles, and eastern Greenland (Fig. 1). K–Ar and Ar–Ar dating of these lavas gives ages ranging from 63 to 54 Myr (refs 14–16). Volcanics exposed subaerially along the Blossville Coast of East Greenland

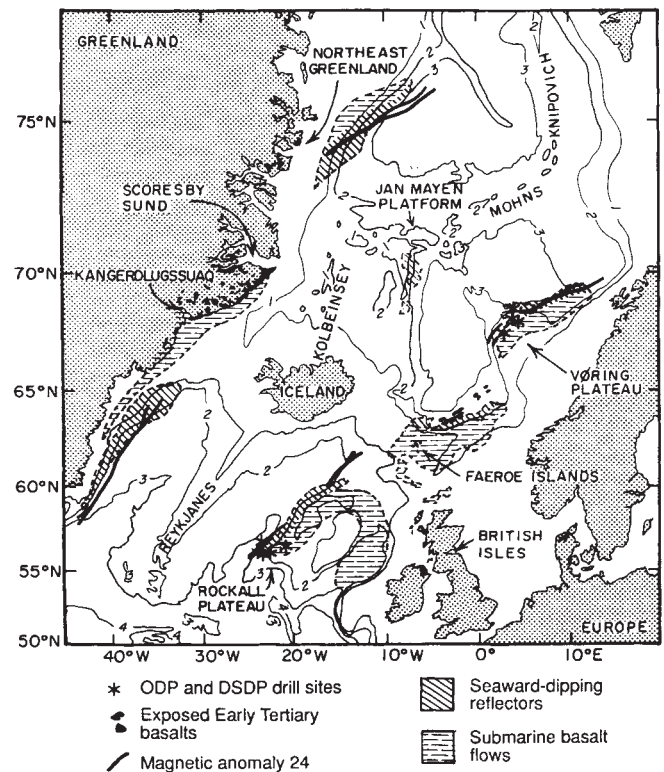


FIG. 1 Map of the North Atlantic region (modified from ref. 10). Shown are locations of subaerial and offshore Early Tertiary volcanic sequences, bathymetry of segments of the Mid-Atlantic Ridge, Iceland, and the Greenland–Iceland–Faeroes Ridge, and position of seafloor magnetic anomaly 24 (54 Myr; ref. 12). Jan Mayen Platform is a bathymetrically shallow region at the southern end of the Mohns Ridge²⁸.

include a high proportion of high-MgO, incompatible-element-enriched lavas in the Kangerdlugssuaq Fjord area to the south, and fractionated Fe–Ti basalts in the Scoresby Sund area to the north. These lavas rest on basement of Precambrian age that was reworked in the north during the Caledonide orogeny. The lower portions of volcanic successions found on the Faeroe Islands and northern British Isles, as well as lavas from Northeast Greenland, chemically resemble material from the Blossville Coast, whereas the upper portions of these succes-

sions consist of lavas similar to modern North Atlantic basalts found along the Reykjanes, Iceland, Kolbeinsey, Mohns and Knipovich segments of the Mid-Atlantic Ridge.

We compiled 288 analyses of primitive lavas ($Mg\# = \text{molar } MgO/(MgO + FeO_{\text{total}}) \geq 0.60$) from Early Tertiary volcanic sequences and modern ocean ridges in the North Atlantic region, some from the literature and some taken from our own work in East Greenland. To aid comparison among these primitive basalt compositions, analyses were corrected for crystal fractionation or accumulation to yield compositions in equilibrium with mantle olivine. Fractionation-corrected TiO_2 and FeO_{total} contents (designated as $TiO_2^{0.70}$ and $FeO_{\text{total}}^{0.70}$) are shown in Fig. 2a for Early Tertiary and modern lavas. Modern North Atlantic, Vøring Plateau, Rockall Plateau and upper British Isles lavas have lower $TiO_2^{0.70}$ and $FeO_{\text{total}}^{0.70}$ than those from Kangerdlugssuaq Fjord, most of the Scoresby Sund section, Northeast Greenland, and the lower portions of the British Isles and Faeroe Islands sections.

Correlations between $TiO_2^{0.70}$ and $FeO_{\text{total}}^{0.70}$ can be produced by mantle melting systematics. The iron content of primary mantle melts increases with mean pressure of melting ($\bar{P}$)^{17,18}. Likewise, the concentration of moderately incompatible elements (such as Na and Ti) decreases as the mean extent of melting ($\bar{F}$) increases. For mid-ocean-ridge basalts (MORBs), a positive correlation between $\bar{P}$ and $\bar{F}$ is inferred¹ from the negative correlation between fractionation-corrected FeO and Na_2O . These systematics can be seen in Fig. 2a in terms of $FeO_{\text{total}}^{0.70}$ and $TiO_2^{0.70}$ for modern North Atlantic basalts, where Iceland corresponds to the highest and Mohns–Knipovich ridges to the lowest $\bar{P}$ and $\bar{F}$. These relationships have been explained by along-strike variations in mantle potential temperature¹. Hotter asthenosphere intersects the solidus at greater depth, resulting in both higher $\bar{P}$ and higher $\bar{F}$.

In contrast to the geochemical relationships found among modern North Atlantic basalts, a strong positive correlation is seen in Fig. 2a between $FeO_{\text{total}}^{0.70}$ and $TiO_2^{0.70}$ for the complete North Atlantic dataset from Early Tertiary to present. The North Atlantic array suggests an inverse relationship between $\bar{P}$ and $\bar{F}$ that cannot be explained solely by variations in asthenospheric temperature with time. Trends with similar orientation are known for restricted ocean-ridge segments and attributed to variability in pooling of mantle melts¹⁹. The magnitude of such effects, however, is small compared with the overall range displayed by the North Atlantic dataset. The striking correlation between $FeO_{\text{total}}^{0.70}$ and $TiO_2^{0.70}$ suggests a more fundamental control on melt generation.

Recent work suggests that the lithosphere may act as a lid on the melting zone^{2,3}. For a thick lithosphere, melting is confined to a shorter path length (lower $\bar{F}$) at greater depth (higher $\bar{P}$). Lithospheric thinning allows asthenosphere to rise further, increasing $\bar{F}$ and decreasing $\bar{P}$. Qualitatively, the North Atlantic array can be understood by changes in lithospheric thickness: at the initiation of magmatism, a relatively thick continental lithosphere existed that rapidly thinned and disappeared by the time of magnetic anomaly 24.

A strong positive correlation is also shown in Fig. 2b between heavy rare-earth element (HREE) fractionation, represented by chondrite-normalized Dy/Yb (denoted $(Dy/Yb)_N$), and $TiO_2^{0.70}$. Because the HREEs are strongly fractionated, yet compatible, in garnet, and relatively incompatible in other mantle minerals, the Dy/Yb ratio is sensitive to the proportion of melting occurring in the presence of garnet. Higher values are expected for higher $FeO_{\text{total}}^{0.70}$ and thus greater $\bar{P}$. We restrict consideration to the HREEs because of the clear evidence of lithospheric contamination of light rare-earth elements (LREEs) in Early Tertiary basalts^{20–23}, which would obscure fundamental features of the melting systematics.

We can quantify the melting systematics required to produce the North Atlantic array seen in Fig. 2b in terms of the distance of asthenospheric upwelling from the solidus to the base of the

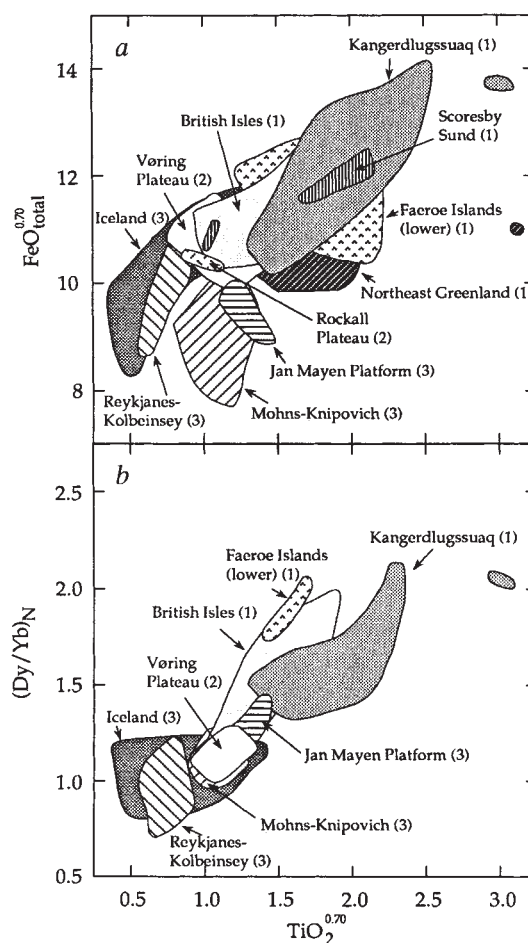


FIG. 2 a, FeO_{total} and TiO_2 contents of 288 fractionation-corrected primitive lavas from Early Tertiary and modern North Atlantic regions. The number following the name of the region indicates the relative age of the lavas: (1) pre-magnetic anomaly 24 age, (2) roughly magnetic anomaly 24 age, and (3) recent. TiO_2 versus $Mg\#$ relationships for each region were examined to determine the $Mg\#$ above which the liquid line of descent slope corresponded to olivine control, typically at $Mg\# \geq 0.6$. Correction for olivine fractionation or accumulation was accomplished by the addition or subtraction of small increments of equilibrium olivine to produce lava compositions with $Mg\# = 0.70$. Highly altered samples were eliminated from the database on the basis of petrographic or hand sample descriptions. Corrected lava compositions are indicated by superscript^{0.70}. Sources of published data are available on request from the authors. b, Dy/Yb normalized to chondritic values (designated as $(Dy/Yb)_N$) versus $TiO_2^{0.70}$ for primitive lavas from the North Atlantic. Rare-earth element data obtained by direct current plasma emission spectrometry and instrumental neutron activation analysis (INAA). For INAA data, Dy values were interpolated from measured Tb and Yb. Note absence of data fields for Scoresby Sund, Rockall Plateau and Northeast Greenland in b due to lack of REE analyses on primitive samples.

lithosphere. We model melting as a polybaric, largely fractional fusion process. For each 1-kbar step of decompression the mantle melts 1.5% by non-modal batch melting. Mantle composition and mineralogy progressively change because of depletion by melting and phase transitions. Compositional arrays of pooled melts formed by decompression melting initiated at different solidus depths are compared in Fig. 3 with the data fields from Fig. 2b. The model results are contoured in terms of the pressure of final melt segregation and $\bar{F}$.

Fields for initial continental rift products (Kangerdlugssuaq Fjord and the lower portions of the British and Faeroe Islands sections) span estimated segregation pressures of 15–25 kbar along melting arrays starting at 40–25 kbar. Estimates of $\bar{F}$ range

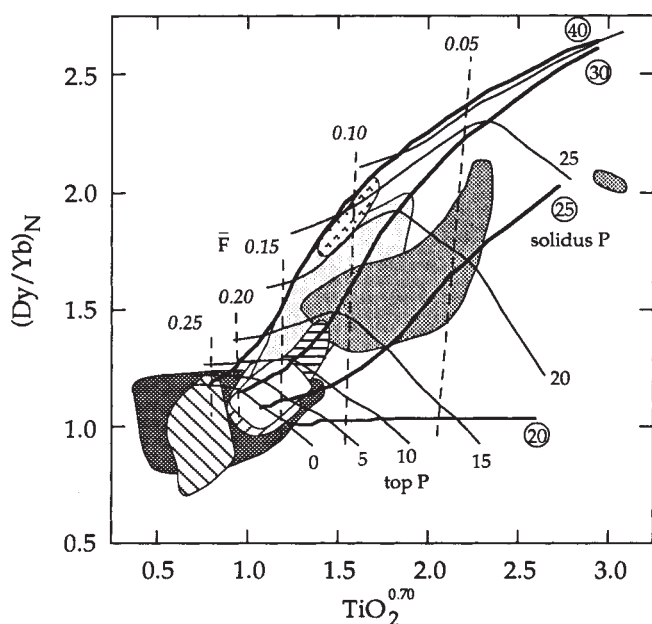


FIG. 3 Model calculations superimposed on data fields from Fig. 2b. Heavy curves show the composition of pooled melts as a function of decreasing pressure for various pressures of solidus intersection, as discussed in the text. Contours of pressure (in kbar) at the top of the melting zone are shown as light curves and contours of F are shown as dashed lines. The mantle source region below the solidus is assumed to have a chondritic Dy/Yb ratio, 0.22 wt% TiO_2 , and a mineral mode of 50% olivine (ol): 25% orthopyroxene (opx): 15% clinopyroxene (cpx): 10% garnet (gt), consistent with estimates of primitive mantle composition^{29,30}. The shape of the melting regime is defined by simple corner flow. For calculations, garnet gradually transforms to spinel between 30 and 20 kbar and spinel (sp) converts to plagioclase (pl) between 14 and 10 kbar (refs 31–33). Melting proceeds by incremental non-modal batch melting³⁴, where melting proportions (ol:opx:cpx:gt:sp:pl) are: 0.10:0.10:0.40:0.40:0.0 between 40 and 30 kbar; –0.10:0.25:0.61:0.20:0.04:0 between 30 and 20 kbar; –0.30:0.40:0.82:0.08:0 between 20 and 14 kbar; –0.18:0.29:0.55:0.04:0.29 between 14 and 10 kbar; and –0.05:0.19:0.28:0.05:0.58 between 10 and 0 kbar. Melting proportions are –0.25:0.30:0.95:0.0:0.0 after aluminous phases are exhausted; and 0.25:0.75:0.0:0.0:0.0 after cpx is exhausted^{31,35}. TiO_2 distribution coefficients are 0.01:0.10:0.20:0.01:0.10:0 and REE distribution coefficients are taken from ref. 36. For each 1 kbar of decompression, 1.5% melt forms and accumulates without further re-equilibration. Residues are recalculated after each increment of melting and adjusted for pressure-dependent phase transitions.

between 0.04 and 0.14. The position of the fields for younger Tertiary lavas from the Vøring Plateau and upper parts of the British Isles section correspond to segregation pressures less than 10 kbar, solidus pressures ≤ 35 kbar, and F greater than 0.12. Similarly, the fields for modern North Atlantic ridge segments also indicate low segregation pressures and even higher F , whereas melting conditions beneath the Jan Mayen Platform seem to be intermediate. Solidus pressure is poorly resolved for modern lavas because almost all of the melting takes place at pressures less than garnet stability, resulting in little or no HREE fractionation for segregated melts. However, recalling Fig 2a, minimum values (~ 20 kbar) are represented by Mohs–Knipovich segments and maximum values (up to 40 kbar) by Iceland.

Temporal and spatial changes in the composition of estimated parental magmas from the North Atlantic suggest that the physical conditions of melting were most strongly controlled by changes in the thickness of the lithosphere and secondarily by changes in mantle potential temperature. Alternatively, to generate the observed ranges in $\text{FeO}_{\text{total}}^{0.70}$, $\text{TiO}_2^{0.70}$ and $(\text{Dy}/\text{Yb})_N$ solely by source heterogeneity would require variations close to the maximum ranges reported for lherzolite xenoliths^{24–26}. We believe it is significant that the gross geochemical features of North Atlantic basalts can be explained readily by changes in the physical conditions of mantle melting and do not require fundamental changes in mantle source composition through time.

It has been argued^{7,8,27} that the high-MgO, incompatible-element-enriched initial rift products found on the North Atlantic margins represent high-degree melts formed in the region of the Iceland plume 60 Myr ago. An intriguing aspect of the results presented in Fig. 3 is the modest extents of melting (<0.14) required to produce parental magmas for the East Greenland, and Faeroe and British Islands volcanics. Previous work on Faeroe Islands basal lavas estimated similar extents of melting (0.08–0.14)²¹. The importance of this result is that the high incompatible-element abundances in these magmas may be the consequence of modest extents of melting rather than large degrees of melting of a highly enriched source⁸. We propose that these low extents of melting are the result of restriction of the length of the melting zone by the presence of a continental lithosphere, which modelling suggests had a maximum thickness of 80–45 km (Fig. 3) during the initial phase of rift magmatism.

Many models regard the Iceland hotspot as integral to the evolution of the North Atlantic^{4–8}. The detection of a strong residual garnet signature in Early Tertiary primitive basalts indicates that mantle melting began at anomalous depth (120–75 km), well within the garnet stability field, in all regions of the North Atlantic where basal lavas are sampled (Fig. 1). This provides geochemical support for models advocating the existence of a region of anomalously hot mantle (that is, plume) beneath the Greenland–European continent at the time of rifting, which has been previously inferred from the geographical distribution of Early Tertiary magmatism^{7,8}. The generation of thick igneous crust during the time of magnetic anomaly 24 along conjugate margins of the North Atlantic basin was the result of rapid lithospheric thinning which outpaced the decay of the asthenospheric thermal anomaly now localized near Iceland. This conclusion, based on geochemical data, is in accord with a lithospheric thinning model⁶ developed to explain crustal thicknesses alone at the time of magnetic anomaly 24. Before this time, the continental lithosphere limited upwelling of asthenospheric mantle, producing basalts that reflect generation by modest extents of melting at high mean pressures. Increased resolution of changes in the nature of the melting regime through time will be provided by the proposed transect drilling by the ODP on the conjugate margins of the North Atlantic basin. □

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Transposon tagging of a male sterility gene in *Arabidopsis*

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TRANSFORMATION of the well-studied maize transposable elements into other plant species¹ should enable transposon tagging methodology² to be used for the isolation of interesting genes in the heterologous host. Here we describe the isolation of a transposon-tagged male sterile mutant in *Arabidopsis thaliana* using the maize *Enhancer-Inhibitor*^{3,4} transposable element system introduced into *Arabidopsis*. The mutant lacks pollen, preventing normal self-fertilization, a characteristic important for production of hybrid seed in many crop plants. We have identified an *Enhancer*-transposase-mediated *Inhibitor* element insertion responsible for the male sterile phenotype, and isolated the corresponding gene named *MALE STERILITY 2*. Critical evidence that the *Inhibitor*-element-containing gene is involved in the male sterile phenotype is provided by the DNA sequences of new excision-derived alleles from independent stable fertile and male sterile progeny of the original mutant.

The maize transposable element system consisting of the autonomous *Enhancer/Suppressor-mutator*(*En*)^{3–5} and non-autonomous *Inhibitor/defective Suppressor-mutator*(*I*) elements has been used for transposon tagging of a number of genes in maize² and was shown to transpose when introduced by transformation into heterologous plant species^{6–8}. We constructed a transformation vector containing three units, namely: (1) a non-mobile *En* transposase source⁶, under control of the strong cauliflower mosaic virus 35S promoter; (2) a mobile *I* element⁹ as insertion mutagen; (3) a hygromycin phosphotransferase gene¹⁰ that confers resistance to the antibiotic hygromycin. This 'in cis two-element *En-I*' vector construct was used for *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis*¹¹. A male sterile plant was found among ten unselected

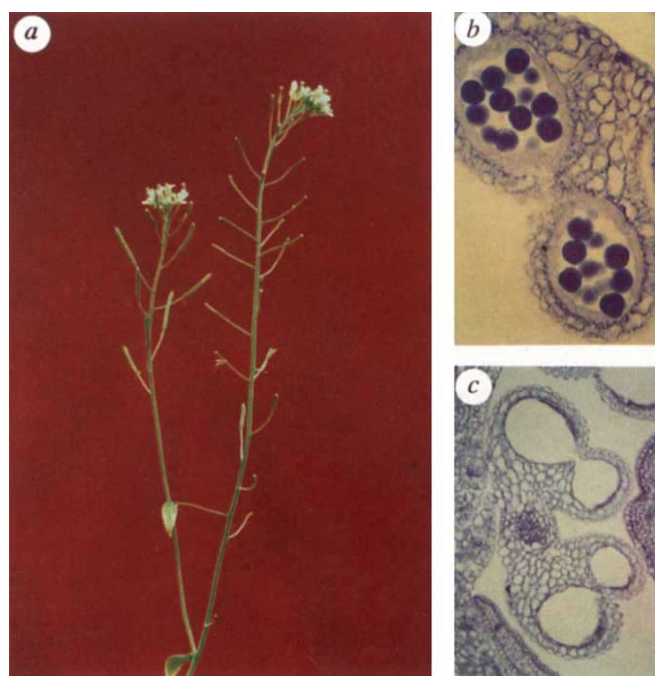


FIG. 1 Male sterile phenotype and pedigree of PA progeny. *a*, The phenotype of a genetically unstable male sterile mutant leading to a chimaeric male sterile/fertile phenotype. As *Arabidopsis thaliana* is a strictly self-fertilizing species, male sterility is characterized by short, empty siliques due to absence of self-pollination (right branch). In one side branch (left) of this otherwise male sterile plant, early excision of an *I* element from the mutated gene has resulted in a reversion from mutant male sterile to wild-type male fertile phenotype (*ms2::I* reverts to *MS2*). Fertile flowers of the revertant sector result in thick, elongated and seed-filled siliques. The plant shown (PA11) is unique among progeny of PA in having such a large revertant sector. In other plants reversion was restricted to single flowers, phenotypically visible as an occasional completely fertilized silique on a male sterile plant. *b*, Cross-section of a wild-type anther shortly before pollen release.

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c, Cross-section of a male sterile anther of similar stage as in *b*. *d*, Pedigree of PA progeny used for subsequent molecular genetic analysis. For the genotypes the presence (*En*) or absence (–) of *En* transposase is shown followed by the *MS2* genotype. R1 and R2 are the revertant sectors of plant F₂ 9–5.

METHODS. A primary transformant (T₁) containing the 'in cis two-element *En-I*' construct (five T-DNA copies at one locus) was self-fertilized and its progeny selected for hygromycin resistance (transposase presence). Among 10 non-selectively grown T₃ progeny (containing 5–10 transposed *I* elements) of one of these T₂ plants, a male sterile plant was found (PA). One inflorescence of PA was outcrossed to the untransformed ecotype *Landsberg erecta*. The rest of the plant was permitted to self-fertilize, yielding only 52 seeds. PA11 was grown from one of these seeds. For sectioning, whole inflorescence tops were embedded in Technovit. Sections (5-μm-thick) were stained with toluidine blue.

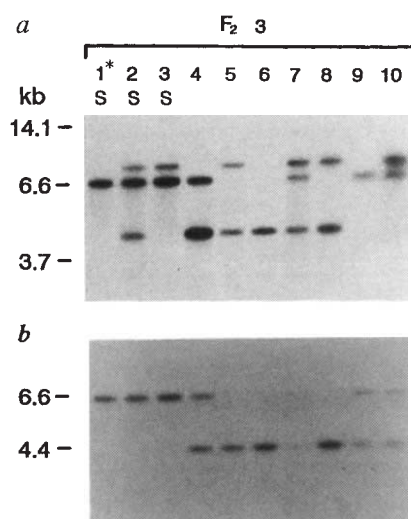


FIG. 2 Segregation analysis of a specific *I* element with the male sterile phenotype. *a*, Segregating F_2 progeny lacking *En* transposase from the cross PA $\times$ Landsberg *erecta* (Fig. 1*d*) was used for hybridization with an *I*-element-specific probe. DNA was digested with *Hind*III, which does not cut inside the *I* element. The F_2 progeny lacks *En* transposase genes, therefore all detected fragments denote stable *I* element inserts. A 6.6-kb *I*-element-containing fragment (■) appears homozygous only in plants displaying a male sterile (S) phenotype. Plant F_2 3-1 (*) has only the *ms2* accompanying *I* element and was therefore used for isolation of the transposon flanking DNA, ultimately yielding a probe for the *MS2* gene. *b*, Homozygosity of plant F_2 3-1 for the *ms2::I* allele was confirmed by rehybridizing the blot with an *MS2*-specific probe. In total, four different F_2 families (43 plants) lacking *En* transposase were analysed (data not shown). Every male sterile plant showed a homozygous *ms2::I* allele.

METHODS. F_2 progenies lacking *En* transposase were grown in the greenhouse, their phenotypes determined and DNA isolated²⁰. *Hind*III-restricted DNA separated by 0.8% agarose gel electrophoresis was transferred using the GeneScreen-Plus nylon membrane protocol. The blot was hybridized with a radiolabelled 0.27-kb fragment specific to the *I* element. Homozygosity of the 6.6-kb *ms2::I*-containing fragment was determined by intensity comparison with other bands in the same lane and confirmed later by rehybridizing the blot with an *MS2*-specific probe (*b*). Rehybridizing is the only way to conclusively establish homozygosity of the *I* homologous fragment in plant F_2 3-1.

ted third generation (T_3) progeny of a successively self-fertilized primary transformant (T_1) with frequently transposing *I* elements. The plant was coded PA (for pollen absent). As the mutation involved can be complemented by *ms1* (data not shown), the only other male sterile mutant without pleiotropic effects described in *Arabidopsis*¹², we called this novel mutated gene *ms2*.

Some seeds (52) were produced after self-fertilization, indicating that the mutant PA phenotype is leaky. Among the PA progeny plants grown from this seed (PA1–PA52), 47 showed a male sterile phenotype, proving that they were *bona fide* self-fertilized progeny. Blot hybridization analysis with an *I*-element-specific probe indicated that the five fertile plants also arose from self-fertilization as they contained at least one homozygous *I*-element-hybridizing fragment (data not shown). Several sterile plants had a few fertile flowers and one plant (PA11) had a completely fertile side branch (Fig. 1*a*). The presence of fertile sectors in otherwise sterile plants indicated that the mutation was probably due to insertion of a transposable element with subsequent excision restoring the activity of the gene involved in male fertility.

Genetic analysis showing 3:1 segregation of fertile: male sterile in progeny of self-fertilized stable heterozygous *MS2 ms2::I* plants and recovery of homozygous male steriles

indicates that the mutant acts sporophytically. In cross-sections of full-grown male sterile anthers, no pollen could be detected (Fig. 1*b* and *c*). In young anthers the tapetal layer appears to be affected, leading to pollen abortion shortly after release from tetrads. Careful examination of the close-contained mutant (without transposase) revealed that flowers developing late in plant growth yield a few seeds, explaining the leaky phenotype. This phenomenon has been reported before for male sterile *Arabidopsis*¹².

To identify the *I* element insertion responsible for the male sterile phenotype, progeny of PA were compared with other families that originated from the same primary transformant and did not segregate for the mutant phenotype by blot hybridization with an *I* element probe. This revealed the presence of a unique *I* homologous band in the PA progeny (data not shown). As PA was hemizygous for *En* transposase, an outcross to an untransformed plant resulted in F_1 plants (Fig. 1*d*), half of which lack transposase (hygromycin-sensitive). All 10 F_1 plants revealed the specific *I* homologous band, suggesting homozygosity in the parent. From four F_1 plants without transposase, the F_2 progeny, segregating 3:1 for fertility: male sterility, revealed the same specific *I* homologous band, which was homozygous in all plants with the male sterile phenotype. Male sterile plant F_2 3-1 contained a single homozygous *I* element, as shown by the unique DNA fragment hybridizing to the *I*

TABLE 1 Nucleotide sequences of the *ms2::I* allele and *En* transposase mediated excision derivatives compared to plant phenotypes

DNA origin	Allele sequences	Genotype	Phenotype
Landsberg <i>erecta</i> (wild type)	ACA AAC ACA AAC	<i>MS2/MS2</i>	fertile
F_2 3-1	ACA AA <u>I</u> A AAC ACA AA <u>I</u> A AAC	<i>ms2::I/ms2::I</i>	male sterile
F_3 9-5R1-4	ACA AAA C ACA AAA C	<i>ms2/ms2</i>	male sterile
F_3 9-5R1-5	ACA AAA C ACA AA <u>I</u> A AAC	<i>ms2/ms2::I</i>	male sterile
F_3 9-5R1-6	ATA AAC ACA AAA C	<i>MS2/ms2</i>	fertile
F_3 9-5R2-2	TTA AAC ACA AA <u>I</u> A AAC	<i>MS2/ms2::I</i>	fertile
F_3 9-5R2-3	TTA AAC ACA AAA C	<i>MS2/ms2</i>	fertile
PA6	ACA AAC	<i>MS2/ms2::I</i>	fertile
PA13	ACA AA <u>I</u> A AAC ACA AAT TAA* C ACA AAT TAA* C	<i>ms2/ms2</i>	male sterile
PA45	ACA AAT TAA* AC ACA AA <u>I</u> A AAC	<i>ms2/ms2::I</i>	male sterile
PA46	ACA AAC	<i>MS2/ms2::I</i>	fertile
F_2 7-7	ACA AA <u>I</u> A AAC ACA AAT TTA AC ACA AAT TTA AC	<i>ms2/ms2</i>	male sterile
F_2 7-10	ACA AA <u>I</u> A AAC ACA AA <u>I</u> A AAC	<i>ms2::I/ms2::I</i>	male sterile

The wild-type Landsberg *erecta* (Ler) sequence shows the trinucleotide target site sequence (AAA) which is duplicated upon insertion of the *I* element shown in plant F_2 3-1. The reading frame codons (determined by cDNA sequence) are separated by gaps. Nucleotides altered in excision derivative alleles if compared to the wild-type sequence are underlined and introduction of a stop codon denoted by an asterisk. Three different origins of the plants analysed (see Fig. 1*d*) enable independent excision events to be isolated. The plants used for excision site sequencing are F_3 plants from two self-fertilized fertile revertant flowers of F_2 plant 9-5 (F_3 9-5R), self-fertilized progeny of the original 'leaky' male sterile mutant (PA) and two F_2 plants (F_2 7-7, 7-10) derived from the cross PA $\times$ Ler. These plants are segregants which lack *En* transposase and contain stable excision derivative alleles that originated in the parental plant (genotypic notation as for Fig. 1*d*). Fertile plants with the wild-type sequence contain at least one other homozygous *I* element, ruling out cross pollination. PCR was performed using primers flanking the *I* element insertion site. Primers were designed based on the sequence of the IPCR fragment from plant F_2 3-1 (*ms2::I/ms2::I*). PCR products were blunt-end cloned in Bluescript SK⁺ (Stratagene). Double-stranded supercoiled plasmid was used for automated sequencing using the *Taq* Dye Deoxy terminator cycle sequencing kit (Applied Biosystems). At least two cloned products of a PCR were sequenced per plant. If the first two sequences were identical, two cloned products of at least one additional PCR were sequenced. The presence of *ms2::I* alleles was determined by hybridizing Southern blots with an *MS2*-specific probe.

GOT	TEA	CTT	AAT	CTT	CTT	TCT	AGT	TAA	TAA	TAT	TCT	TGT	TGC	-71	5	-	ATC	TGT	GTT	CTT	-61
Met	GlU	Ala	Leu	Phe	Leu	Ser	Ser	Ser	Ser	Ser	Ser	Val	GlU	Val	GlU	Val	Asn	Lys	Leu	Thr	20
ATG	GAG	GCT	CCT	CCT	TGT	AGT	TCT	TCT	TCT	TCC	TCC	TCT	TCA	GGG	TCA	AAG	Asn	Ala	GAT	ACT	60
Arg	Leu	His	Asn	His	Cys	Val	Trp	Trp	Thr	Val	Ala	Arg	Asp	Arg	Ala	Asp	Phe	Gly	Pro	120	
AGG	TTA	CAC	AAC	CAT	TGT	TGT	TGG	TCT	AGA	GTG	ATT	Arg	GAG	Ala	Ala	Asp	Thr	GCT	CCC	40	
Thr	Trp	Gly	Arg	Val	GlU	GlU	GlU	GlU	Asp	GAT	GAT	Gly	Asn	Asn	Asn	Ala	Alu	Ser	Pro	180	
ACT	TGG	TGC	CGT	GTA	GGT	GGT	GGT	GGT	ASP	GAT	GAT	GGG	AGA	Asn	Asn	Ala	GCA	GAG	ACT	60	
Ile	Arg	Val	Ser	Ser	Leu	Val	Ala	Asp	Arg	Gly	Gly	Gln	Val	Ile	Arg	Gln	Ser	Gln	Ser	240	
ATT	CGG	GTT	TCT	TCT	CGC	CTT	TTG	AAA	AGA	AGA	GCT	CAG	TGA	CTG	ATT	AGG	GAA	CAG	AGT	80	
Pro	GCT	Met	Asp	Ala	Gln	Ala	Thr	Leu	Val	Ala	Trp	Pro	Asn	Ala	Gly	Asp	Arg	Thr	Ile	100	
CCG	GCT	ATG	GAT	GCT	GAG	CTA	TTG	GTT	CTG	Trp	CCA	AAc	GGG	Asn	Gly	Asp	ACC	ATT	GAG	100	
ATC	AAT	GGA	GTA	Val	Arg	Ala	Trp	Leu	Met	Pro	Phe	Ser	Met	Val	Alu	Gly	Met	Lys	Gln	120	
CTG	Leu	Gly	Ile	Ile	Ser	Phe	Leu	Gln	Gly	Lys	Lys	Leu	Ile	Trp	Ala	GCT	Asp	Arg	GAG	140	
GGA	CTT	GCC	ATT	ACT	AGT	CTT	CTC	Ala	GGG	Ala	GGG	Ala	TTT	CTC	Ala	GCT	GCC	Arg	GAG	140	
Phe	Thr	Ala	Lys	Val	Leu	Ile	Glu	Lys	Val	Tyr	Arg	Met	Ala	Pro	Asp	Val	Arg	Ser	Lys	160	
TTA	GCT	Ala	ATA	CTA	Cys	Arg	TGT	GAG	Ala	ATC	GCT	CCT	GAT	ATC	GAT	ATC	Ala	Ala	Ala	180	
Tyr	Leu	Trp	TTG	ATT	Ala	GCA	CCC	Ala	AGC	Ala	GAA	GCT	GGC	ATC	GAG	CGG	CTA	Ala	GAG	180	
Met	Asp	Alu	Glu	Leu	Phe	Asn	Thr	Ala	Lys	Glu	Thr	His	Ala	Ala	Ala	Ser	Tyr	Met	GAG	200	
TTA	GAT	GCA	GAG	CTT	TTT	ATT	ACT	CTA	Ala	GAA	GAG	ACT	CGA	GCA	GCA	TCT	TAC	Arg	TCT	200	
ATG	Trp	Thr	Lys	CTC	ATC	ACT	CTT	GTG	Ala	CCG	Ala	Ala	Cys	Asp	Trp	Ala	Gly	Gln	Gln	220	
Ala	GCA	GAT	Asn	GCT	GAA	GAG	ATT	CGC	Ala	GAA	GTT	GAT	TTT	ATA	ATC	Ala	TCT	GCT	CAc	240	
Thr	Thr	Phe	Asn	Gln	Arg	Tyr	Asp	Val	Ala	Gly	Val	Ala	Ala	Ile	Asn	Ser	Ala	Ala	Ala	260	
ACA	ACC	TTT	Ala	Gly	Ala	GAG	TAC	GAT	GTT	GCT	CTG	GAC	Alc	Alc	Alc	Alc	GGG	CCC	GGT	280	
CTC	ATG	GGA	TCT	GCC	Ala	Lys	TGC	Ala	Lys	GCT	CTA	AAA	CTC	TTC	Trp	CAA	GTA	TCC	CAA	280	
Tyr	Val	Asn	Gly	Gln	Arg	Gly	Gln	Ala	Arg	Ile	Met	Gly	Lys	Pro	Phe	Ser	Met	Gly	Asp	300	
TAT	GTG	AAT	GGA	CAA	GAG	CAA	GGA	AGG	ATC	ATG	GAG	Ala	GAG	Ala	TTT	TCT	ATC	GGA	GAT	300	
Ile	Ala	Thr	Ala	Asn	Phe	Leu	Glu	Ala	Gly	Asn	Arg	Lys	Ala	Leu	Asp	Val	Asp	Arg	Glu	320	
Lys	GCA	ACA	GAG	Alc	TTC	CTC	GAA	GGA	AGA	AGA	Ala	GCA	TAT	GAT	GTT	GAT	AGA	GAG	Ala	340	
Ala	Leu	Ala	Gly	Ala	Ala	Lys	Gly	Thr	Gln	Ala	Gln	Ala	GAT	GAT	GAT	GAG					

FIG. 3 Nucleotide and predicted amino-acid sequences of *MS2* cDNA. Nucleotides are numbered starting from the first position of the ATG initiation triplet. A nonsense codon upstream (-36) indicates that this is the translation start. The polyadenylation signal sequence (position 1,967; underlined) precedes the poly(A) tail. The position of the *I* element insertion (AAA) at position 1,800 is underlined. The last three amino-acids (Gly-Arg-Ala) contain a putative C-terminal microbody targeting signal (CMTS) suggesting a role for this part of the protein which is absent in male sterile mutant alleles as a result of excision footprints causing frameshifts (Table 1). Database searches (FASTA/BLAST) reveal homology to a DNA sequence (EMBL release 33.0, 12/92) located upstream of the wheat mitochondrial 26S ribosomal RNA *rrn26* gene. The region of homology in the *MS2* cDNA (1,040–1,203) is underlined and shows 77.6% identity to the wheat mitochondrial nucleotide sequence. This region is located about 2 kb upstream of the *rrn26* gene at positions 650–814 of the sequence²¹. No function has been ascribed to this mitochondrial DNA segment, which has an open reading frame whose predicted amino acids show 81.7% identity to the *MS2* protein (55 amino acids). The homology in the mitochondrial DNA segment does not extend beyond this region but is precisely flanked by intron-exon boundaries.

METHODS. The cDNA clone (largest out of 3 sequenced) was isolated by screening an *Arabidopsis thaliana* ecotype Landsberg *erecta* flower-specific cDNA library (frequency 1/12,000). Both strands of the cDNA were sequenced (as described in Table 1 legend). Subclones of specific restriction fragments and specific oligonucleotide primers were used to obtain the complete sequence. Sequence was analysed using the Genetics Computer Group (GCC) software package²².

probe (Fig. 2a). DNA from this plant was used to isolate the transposon flanking DNA by inverse polymerase chain reaction (IPCR)^{6,13}. The IPCR fragment was cloned, sequenced and used as a probe to isolate clones from a flower-specific complementary DNA¹⁴ λ library. DNA sequencing revealed that the *I* element insertion was in the 3' end of the codogenic region of the cloned gene we called *MALE STERILITY 2 (MS2)*. Rehybridization of the F₂ progeny blot with an *MS2* gene probe confirmed that the specific *I* element was homozygous in all male sterile plants (Fig. 2b). We identified plants hemizygous for *En* transposase (*En*/- in Fig. 1d) which were chimaeric for male sterility/fertility, either in somatic sectors or in progeny. The excision products from the selfed progeny that lacked transposase were isolated and sequenced (Table 1). In all male sterile plants either the inserted *I* element was still present, or excision led to frameshift mutations. The fertile plants contain a 'revertant' allele which is either wild-type or an 'in-frame' excision footprint, restoring the proper reading frame. In maize, one out of ten excision events leads to an 'in-frame' excision without leaving additional nucleotides¹⁵, yet these infrequent events are present here in every plant with a revertant phenotype (dominant allele). The excision derivative alleles, defined by their nucleotide sequence, can explain the phenotype for every plant. These independently derived 'new' alleles (minimal 6), which determine the male sterile/fertile phenotype, demonstrate conclusively that this *I*-element-tagged gene is responsible for the male sterile mutation. The *MS2* gene cDNA sequence (Fig. 3) reveals a short region of homology with an open reading frame in the wheat mitochondrial genome. The relevance of mitochondria and respiration to pollen fertility has been well established¹⁶. Also, rearrangements in the mitochondrial genome are responsible for 'cytoplasmic' male sterility. Thus homology associated with the *MS2* gene now provides a clue to its biochemical function.

These results establish tagging of a novel male sterility gene (*MS2*) by correlation of phenotype with genotype at the DNA sequence level and subscribe the further use of transposon tagging with heterologous transposons. To our knowledge, this is the first isolated gene determining a nuclear male sterile mutant (monogenic recessive) phenotype. Such sporogenous male sterile mutants with impaired microgametogenesis have been described in many plants¹⁷. Further characterization of the *MS2* gene and homologous genes from other species will increase our knowledge on pollen development¹⁸ and provide methods for the production of male sterile crop plants that will be useful in hybrid seed production¹⁹. □

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Numerous candidate plasticity-related genes revealed by differential cDNA cloning

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PLASTICITY is a property of the nervous system that allows it to modify its response to an altered input. This capacity for change suggests that there are molecular mechanisms in neurons that can couple stimuli to long-term alterations in phenotype¹⁻³. Neuronal excitation elicits rapid transcriptional activation of several immediate-early genes⁴, for example *c-fos*, *c-jun* and *zif268*. Many immediate-early genes encode transcription factors that control expression of downstream genes whose products are believed to bring about long-term plastic changes^{3,4}. Here we use a highly sensitive differential complementary DNA cloning procedure to identify genes that may participate in long-term plasticity. We cloned 52 cDNAs of genes induced by the glutamate analogue kainate in the hippocampus dentate gyrus. The number of these candidate plasticity-related genes (CPGs) is estimated to be 500–1,000. One of the cloned CPGs (*16C8*), encoding a protease inhibitor, is induced by a stimulus producing long-term potentiation and during dentate gyrus development; a second, *cpg1*, is dependent on activation of the NMDA (*N*-methyl-D-aspartate) receptor for induction and encodes a new small, dentate-gyrus-specific protein. Seventeen of the cloned CPGs encode known proteins, including six suggesting that strong neuronal activation leads to *de novo* synthesis of vesicular and other synaptic components.

As a source of CPG transcripts we chose the hippocampus dentate gyrus (DG), a brain area that participates in plasticity events such as long-term potentiation (LTP)⁵, seizures and kindling^{6,7}, and is involved in learning and memory⁸. To induce CPGs in the DG, rats were injected with kainate, a treatment that induces seizures and LTP-like potentiations⁷. Optimal CPG induction was determined by monitoring kainate effects on proenkephalin messenger RNA levels. Proenkephalin is a suitable marker for CPGs, being encoded by a downstream gene that is controlled by previously induced immediate-early genes, and is induced in the DG by LTP¹⁰ or seizures¹¹.

CPG cDNAs were operationally defined as complementary to transcripts induced in the DG six hours after kainate treatment. To clone these, we constructed a subtracted cDNA library from the DG of kainate-injected rats, and differentially screened it for kainate-induced cDNAs (Fig. 1a). To overcome the limited sensitivity of differential screening, a highly sensitive protocol was used (Fig. 1b); allowing detection of ~80% of the library clones.

Duplicate Southern blots containing 25 clones screened as described (Fig. 1b legend) are shown in Fig. 1c: arrows indicate potential CPG cDNAs, whose inserts produced a stronger signal with the kainate-activated probe (upper blot, +), than with the control probe (lower blot, -). Occasionally clones that appeared to be downregulated by kainate were observed, but these were not investigated further. Induction of positive clones (two independent screens) was confirmed by northern blotting. Examples are shown for three clones marked as kainate-activated (Fig. 1c) alongside three other clones and glyceraldehyde 3-phosphate dehydrogenase (G3PD)/proenkephalin controls (Fig. 1d). So far, 1,000 clones of the subtracted kainate-activated DG library have been screened: 52 clones (~5%) were of CPGs and were partially sequenced, one third were of known genes (Table 1), and the remainder were new.

LTP is the most popular model for memory-related synaptic

TABLE 1 Known CPGs and their possible relationship to plasticity

Clone	Product	Function and possible relationship to plasticity	Ref.
Immediate-early genes			
230	c-Jun	Transcription factor	4
284	zif/268	Transcription factor	4
441	c-Fos	Transcription factor	4
514	FosB	Transcription factor	4
639	CREM	Transcription repressor	23
Differentiation and growth response			
263	MyD118	Gene inducible by IL-6 and LIF in myeloid cell differentiation	14, 24
403	16C8	Fibroblast growth factor responsive gene, encodes protease inhibitor TIMP	18, 20
730	ME491	Melanoma associated antigen	25
913	Tyrosine phosphatase	Placenta and brain tyrosine phosphatase	15, 16
Heat-shock proteins			
271	Hsp70	Heat-shock protein stabilizes newly synthesized proteins	17
450	Hsp27	Small heat-shock protein	26
Membrane-, vesicle- and synapse-related			
132	Clathrin heavy chain	Membrane and vesicle traffic	27
180	Secretogranin	Secretory vesicle protein	28
501	Dynorphin	Neuropeptide	29
575	Hsc70	Heat-shock protein homologue involved in uncoating of clathrin from coated pits	30
618	Syndecan	Heparan sulphate proteoglycan core protein	31
777	COMT	Catechol-O-methyl transferase, a catecholamine degrading enzyme	32

All 52 CPG cDNAs were partially sequenced, from both ends and subjected to FASTA searches for sequence homologies. The 17 clones shown shared homologies with the genes indicated. The numbers designating the clones represent their identification numbers during screening. References for the DNA sequences and of possible relationships to plasticity are given in the last column.

plasticity⁵. In most cases of LTP, and in other plasticity paradigms^{2,12}, glutamate NMDA receptors are thought to mediate induction while non-NMDA receptors participate in maintenance of plasticity⁵. Figure 2a shows sample *in situ* hybridizations with two of the new CPGs: *cpg1*, whose induction is blocked by the NMDA-receptor antagonist MK-801, and *cpg2*, whose induction is independent of the NMDA receptor. Induction of 40% of the 52 cloned CPGs depends strictly on NMDA receptor activation (results not shown). CPG 403 (*16C8*; Table 1) was induced in the DG six hours after induction of LTP. Induction levels ranging between those observed for rats 1 and 2 (Fig. 2b) were seen only in the DG on the stimulated side (I) and not on the contralateral (C) side. The same CPG (*16C8*) is also expressed during DG development (Fig. 2c).

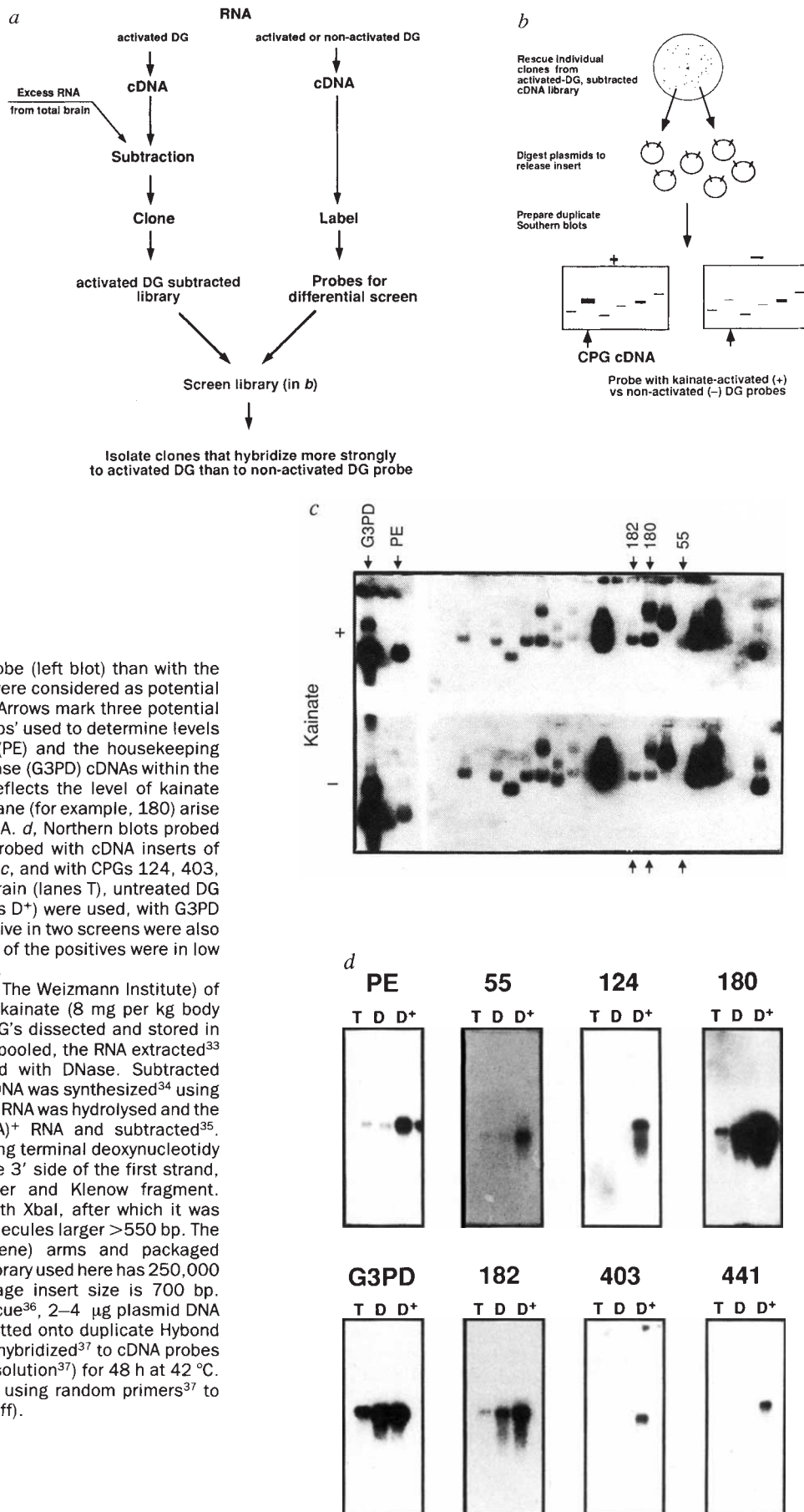
One of the new CPGs, *cpg1*, was highly specific to the adult activated DG (Figs 2a, 3a), and was dependent on NMDA-receptor activation (Fig. 2a) for induction; this CPG was chosen for further analysis. The entire sequence of the major *cpg1* transcript (4,207 base pairs) was determined. The open reading frame (Fig. 3b), predicts a small basic protein containing a repeated 6-amino-acid motif (Fig. 3b, asterisks). In addition, it contains two cysteine residues at positions 73 and 83, preceded by three amino acids (E, T and K at positions 70–72) that are conserved in the neurotrophin family¹³ (Fig. 3c).

A surprisingly large number of CPGs have been isolated, and the total number of CPGs can be estimated as a result of the high

FIG. 1 CPG cDNA cloning approach. *a*, Scheme of subtractive and differential cDNA cloning strategy. Poly(A)⁺ RNA was extracted from the DG of kainate-treated animals or control animals and used for first-strand cDNA synthesis. Ubiquitous activated DG cDNA sequences were subtracted by hybridization to excess poly(A)⁺ RNA from total rat brain. The subtracted cDNA was then used to construct a library. First-strand cDNA from activated and non-activated DG was also radioactively labelled to use as probes in the differential screen. Probes were not subtracted to avoid screening artefacts resulting from variability in subtraction efficiency. *b*, Clone-by-clone screening procedure. The two main features of the screen were: (1) individual plaques of the (unamplified) library were picked and 'rescued' as plasmids (pBluescript) from the λ ZAP vector. Plasmids were restricted to release the cDNA insert and run on duplicate agarose gels; each lane contains DNA of an individual clone. Gels were Southern-blotted and differentially screened for kainate induction. (2) cDNA probes used for the differential screen were labelled to high specific radioactivity and used at very high concentrations. Clones producing a

stronger signal with the activated DG probe (left blot) than with the control non-activated probe (right blot), were considered as potential CPGs. *c*, Example of differential screen. Arrows mark three potential CPG clones. On the left are Southern 'strips' used to determine levels of the kainate-inducible proenkephalin (PE) and the housekeeping glyceraldehyde 3-phosphate dehydrogenase (G3PD) cDNAs within the two probes. The PE/G3PD signal ratio reflects the level of kainate activation. Clones giving two signals per lane (for example, 180) arise from incomplete digestion of plasmid DNA. *d*, Northern blots probed with potential CPG clones, as indicated by arrows in *c*, and with CPGs 124, 403, 441. Poly(A)⁺ RNA (3 μ g) from total rat brain (lanes T), untreated DG (lanes D) and kainate-activated DG (lanes D⁺) were used, with G3PD and PE acting as controls. All clones positive in two screens were also positive on northern blots; however, 15% of the positives were in low abundance, making visualization difficult.

METHODS. DG RNA: Male rats (Wistar of The Weizmann Institute) of 8–10 weeks old were injected i.p. with kainate (8 mg per kg body weight), killed 6 hours later, and their DG's dissected and stored in liquid N₂. DG's from ~100 animals were pooled, the RNA extracted³³ and selected for poly(A)⁺, then treated with DNase. Subtracted activated-DG cDNA library: First-strand cDNA was synthesized³⁴ using oligo(dT)–XbaI primer adapter (Promega). RNA was hydrolysed and the cDNA mixed with total rat brain poly(A)⁺ RNA and subtracted³⁵. Second-strand cDNA was synthesized using terminal deoxynucleotidyl transferase to add an oligo(dA) tail to the 3' side of the first strand, and then oligo(dT)–XbaI primer adapter and Klenow fragment. Double-stranded cDNA was restricted with XbaI, after which it was selected on an agarose gel to include molecules larger >550 bp. The cDNA was ligated into λ ZAP (Stratagene) arms and packaged (Gigapack, Stratagene). The subtracted library used here has 250,000 PFU (85% recombinants) and the average insert size is 700 bp. Screening: After pBluescript plasmid rescue³⁶, 2–4 μ g plasmid DNA was digested with XbaI and Southern-blotted onto duplicate Hybond N⁺ filters (Amersham)³⁷. The filters were hybridized³⁷ to cDNA probes (5 $\times$ 10⁷–10⁸ c.p.m. per ml hybridization solution³⁷) for 48 h at 42 °C. First-strand cDNA (100 ng) was labelled using random primers³⁷ to give a probe of 1.2 $\times$ 10⁹ c.p.m. (Cerenkoff).



sensitivity of the screen. Considering that CPGs comprise 5% of the clones screened, and a complexity of ~20,000–30,000 DG RNA species, the total number of CPGs can be extrapolated to 500–1,000. This number may reflect the complex processes induced by activity in DG neurons.

How could the 17 known cloned CPGs (Table 1) contribute to long-term plasticity changes? They can be classified into several categories. The immediate-early genes are induced by stimuli causing plasticity changes⁴. Genes that are induced in other systems during growth or differentiation responses: for example, interleukin-6 and leukaemia inhibitory factor (LIF) induce MyD118 (clone 263) in myelocytes and are also inducers of neuronal differentiation¹⁴. Tyrosine phosphatases (clone 913) are implicated in development of the nervous system^{15,16}. The two heat-shock proteins may relate to increased protein

synthesis¹⁷, or to stress effects of kainate. The growth-factor-inducible 16C8 (clone 403; Table 1) encodes a protease inhibitor¹⁸. This CPG is induced in the DG by LTP-producing stimuli and during plastic phases of DG development¹⁹ (Fig. 2). Tissue-plasminogen activator protease has been reported to be induced by LTP-producing stimuli²⁰. The interplay of proteases and their inhibitors is implicated in mechanisms of neurite growth²¹, a possible feature of long-term plasticity^{2,3,5,7,21}.

The most interesting group of known CPGs is that classified as membrane-, vesicle- and synapse-related (Table 1). This sample, although small, indicates that long-term plasticity mechanisms operating under our chosen conditions may involve increased synthesis of presynaptic vesicles (clathrin heavy chain, dynorphin, secretogranin and Hsc70), as well as other synaptic constituents (COMT, syndecan).

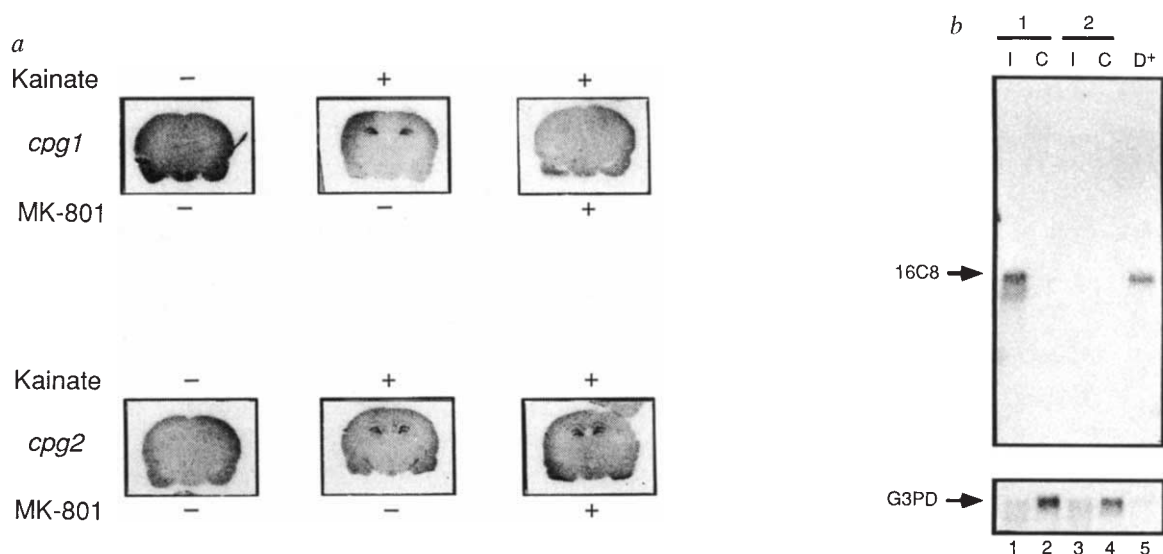


FIG. 2 NMDA receptor, LTP and developmental induction of CPGs. *a*, *In situ* hybridizations of two novel CPGs to rat brain sections. Untreated rats (left sections), rats treated with kainate (middle), or rats treated with kainate and MK-801 (right) were killed 6 h after injection. Coronal cryostat sections (15- μ m) were cut from the brains and hybridized³⁸ with ³²P-labelled DNA of clones 406 (*cpg1*, upper panel) and 239 (*cpg2*, lower panel). MK-801 treatment was achieved by injecting rats 30 min before the kainate injection with 5 mg per kg body weight of MK-801 (RBI, Natick, MA). In some experiments, MK-801 injection was repeated 2 h after kainate injection. *b*, Induction of CPG 403 by an LTP-producing stimulus. LTP was evoked in 6 anaesthetized rats. Six hours after perforant path stimulation, the ipsilateral (I; lanes 1,3) and contralateral (C; lanes 2,4) DG were dissected out separately and RNA isolated. Material from two rats (rat 1, lanes 1 and 2; and rat 2, lanes 3 and 4) are shown. Each lane contains total RNA (7–10 μ g) from one DG. Lane 5 (D⁺) contains 1 μ g poly(A)⁺ RNA from kainate-stimulated DG. Blot was probed with clone 403 (16C8; top autoradiogram) and subsequently, to normalize for RNA amounts, with G3PD (lower autoradiogram). Secretogranin (clone 180; Table 1) levels did not change under the same conditions of LTP induction. These two clones were chosen first for LTP-analysis because of their high level of expression in the activated DG. *c*, CPG 403 is expressed in the developing DG. Poly(A)⁺ RNA (3 μ g) from kainate-activated adult DG (lane 1; AD⁺), non-activated adult DG (lane 2; AD), DG of 21-day-old rats (lane 3; p21), DG of 14-day-old rats (lane 4; p14) and whole hippocampus (the DG is not discernible at this stage) of 7-day-old rats (lane 5; p7) were analysed on a northern blot. The blot was probed first with CPG 180 and then with CPG 403 (16C8) cDNA inserts.

METHODS. The perforant path was stimulated by a stereotactically positioned electrode in 6 anaesthetized rats. Field potentials evoked by low-frequency stimuli (1–10V, 0.1 ms) were recorded extracellularly before and after inducing LTP. LTP was elicited by 10 consecutive high-frequency trains (0.1-ms pulses of 1,000 μ A delivered at 400 HZ for 50 ms). After 30 min, the increases in the slopes of the field

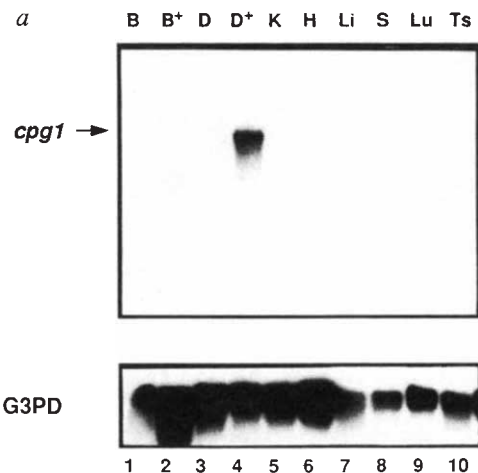
excitatory postsynaptic potentials (e.p.s.ps) and in the population spikes were 30–100% and 100–300%, respectively. Rats 1 and 2 gave maximal increases of 95 and 59% in e.p.s.p. slopes, respectively. The variability in CPG 403 induction level among individual animals could stem from variability in the number of fibres tetanized, from varied response levels, or from effects of anaesthesia⁵ in different animals.

FIG. 3 Characterization of the novel CPG *cpg1*. **a**, Tissue distribution. Northern blot containing 5 μ g poly(A)⁺ RNA from total rat brain of untreated (B), kainate-treated animals (B⁺), untreated DG (D), kainate-activated DG (D⁺), kidney (K), heart (H), liver (Li), spleen (S), lung (Lu), and testis (Ts) was probed with clone 406 (*cpg1*) cDNA and subsequently with a G3PD probe (lower panel). **b**, Predicted amino-acid sequence of the *cpg1* product. Over 30 independent cDNA clones were mapped and used to determine the sequence of the major *cpg1* transcript and a few minor variants. There were at least 4 modes of alternative splicing in the 5' untranslated region, none of which affected the predicted open reading frame. The sequence of the open reading frame and the predicted protein sequence are shown in single-letter amino-acid code. Two pairs of basic amino acids are underlined. A repeated amino-acid motif (PSVI[R/H]P) is indicated by asterisks. **c**, Cysteine residues conserved between the neurotrophin family and the predicted *cpg1* product. Sequences of the nerve growth factor (NGF), neurotrophin (NT3) and brain-derived neurotrophic factor (BDNF)¹³ are compared to the predicted *cpg1* product (CPG1) at residue positions 70–83. Cysteines are underlined and the conserved preceding 3 amino acids are shown in bold.

METHODS. To obtain clones spanning the full-length *cpg1* transcript, two additional (non-subtracted) cDNA libraries were constructed from activated DG RNA as described for Fig. 1. One library was constructed using internal primers derived from sequences at the 5' side of the original clone 406 and was selected to include cDNAs larger than 2 kb. The second library was constructed with an oligo(dT)–*NotI* primer adapter (Promega) in order to span artificial termination of clones resulting from internal *XbaI* site(s). Screening the activated and size-selected DG libraries with clone 406 resulted in 179 positives. Clones spanning the entire sequence were isolated and sequenced on both strands using Sequenase (USB).

Kainate causes not only seizures and potentiations but also glutamate excitotoxicity^{7,22}. The DG, however, is the hippocampal region least prone to damage by kainate²². Furthermore, 15 of the 17 known CPGs cloned (Table 1), appear to be relevant to plasticity. First, the immediate-early genes are induced by plasticity-producing stimuli⁴; second, the differentiation and proliferation response genes are not associated in other systems with cell death; finally, the vesicle and synapse building blocks do not implicate neuronal toxicity but rather synapse remodeling.

Our study describes highly sensitive differential cDNA cloning of a large number of CPG cDNAs, and predicts the existence of ten times as many more. The CPGs include known genes (for example, those encoding 16C8, secretogranin, clathrin heavy chain) and new genes (such as *cpg1*) not previously considered as potential participants in long-term plasticity. The extent of overlap between the different mechanisms underlying various CNS plasticity phenomena is not known^{2,3}, but one way to clarify this issue would be to define the panel of CPGs induced in each case. □



b

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ATGCCCAAGAGTCACATCCTTACCTACACTGGTATAAGAACGAAACGGCCCATCAGTA
1  M A K K S H P Y L H W Y K N E N G P S V 20
          *****
ATCAGGCTGGTGGAGGAGAGGTTTCAGGGAGCCCTAGCTCTACCGCTGCTCTCTTTGTC
21  I R P G G R R G S G S P S S T A A L F V 40
          *****
TCTGTAGATGCAAGCAGAGGGCGAATACCTTACGTTATACACCCCTTGGTGACCAAA
41  S V D A S R G R T L P S V I H P L V T K 60
          *****
TCTAAGGTCACACAGATGGTCTGGTGAACGAAATGCTCATTAAACCAACCCCAAGC
61  S K G H T D G P G E T K C S L N Q T P S 80
          *****
CACGTTTGTGGAAAGGAAGTCTTAGGGACAAGGGGAGGGGGA
81  H V C G K E L L G T R G G G 94
  
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c

NGF:	ETK <u>C</u> RAPNPVES <u>G</u> C
NT3:	ETR <u>C</u> KEARPVKNG <u>C</u>
BDNF:	ETK <u>C</u> NPMGYTKEG <u>C</u>
CPG1:	ETK <u>C</u> SLNQTPSHV <u>C</u>

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Characterization and localization of the *FMR-1* gene product associated with fragile X syndrome

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THE fragile X syndrome is the most frequent form of inherited mental retardation after Down's syndrome, having an incidence of one in 1,250 males^{1,2}. The fragile X syndrome results from amplification of the CGG repeat found in the *FMR-1* gene³⁻⁶. This CGG repeat shows length variation in normal individuals and is increased significantly in both carriers and patients³⁻⁶; it is located 250 base pairs distal to a CpG island⁶ which is hypermethylated in fragile X patients⁴⁻⁷. The methylation probably results in downregulation of *FMR-1* gene expression⁸. No information can be deduced about the function of the *FMR-1* protein from its predicted sequence. Here we investigate the nature and function of the protein encoded by the *FMR-1* gene using polyclonal antibodies raised against the predicted amino-acid sequences. Four different protein products, possibly resulting from alternative splicing, have been identified by immunoblotting in lymphoblastoid cell lines of healthy individuals. All these proteins were missing in cell lines from patients not expressing *FMR-1* messenger RNA. The intracellular localization of the *FMR-1* gene products was investigated by transient expression in COS-1 cells and found to be cytoplasmic. Localization was also predominantly cytoplasmic in the epithelium of the oesophagus, but in some cells was obviously nuclear.

As a first step in the identification and characterization of the *FMR-1* gene product, antibodies were raised against different regions of the predicted amino-acid sequence of the *FMR-1* protein³. Two different methods were used. A complementary DNA fragment of *FMR-1* containing nucleotides 940-1,325 was cloned in the *Escherichia coli* expression vector pGEX⁹ and antibodies were raised in rabbits against the *FMR-1* fusion protein ($\alpha 765$). The second approach was to use a synthetic oligopeptide corresponding to the carboxy-terminal end (position 632 to 656) of the *FMR-1* protein³ as antigen ($\alpha 1079$)¹⁰.

These antibodies were then used to analyse the *FMR-1* protein (FMRP) in lymphoblastoid cell lines from patients ($n=5$) and controls ($n=3$). FMRP was immunoprecipitated with $\alpha 765$ and analysed by immunoblotting. Four species (M_r s 74K, 72K, 70K and 67K) (Fig. 1A) that were present in the controls were absent in four of five patients. The lack of cross-reactive

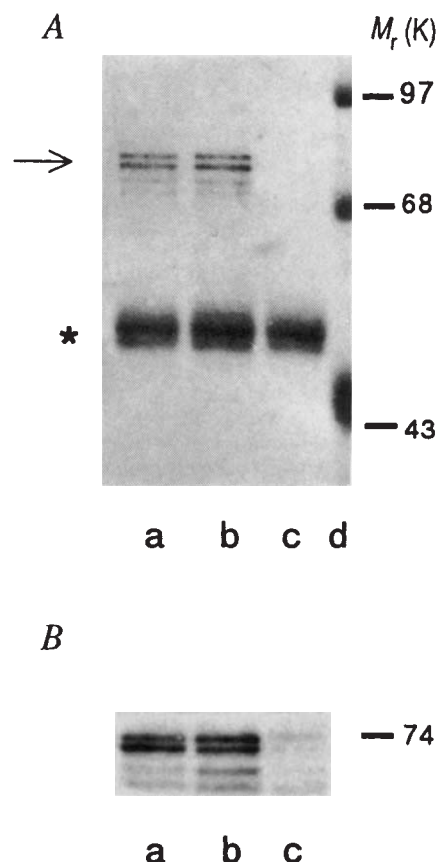


FIG. 1 Immunoprecipitation of lymphoblastoid cell lines with $\alpha 765$ antibodies. A, Control cells: lanes a (C49) and b (ROS31) and fragile X patient cells in lane c (RJK1411). Sizes of the protein bands are 74, 72, 70 and 67K. Lane d, molecular weight standards. Nonspecific band (IgG) is indicated by an asterisk, the position of FMRPs is indicated by an arrow. B, Control cells: lane a (ROS31) and b (GM7381), cells from a mosaic patient lane c (RJK2368). Molecular weight of upper band indicated on the left.

METHODS. Polyclonal antibodies were raised in rabbits against a GST (glutathione S-transferase)-*FMR-1* fusion protein obtained using the pGEX plasmid expression system⁹. A cDNA fragment containing nucleotides 940 to 1,325 of the coding and 3,557-3,765 of the non-coding sequence of the previously published *FMR-1* cDNA³ was cloned in the *EcoRI* site of pGEX-3X. Expression of the construct in the protease-deficient *E. coli* strain B121 resulted in the synthesis of a *FMR-1* polypeptide of 134 amino acids fused with the C terminus of Sj26, a 26K glutathione S-transferase. The *FMR-1* polypeptide consisted of amino acids 314 to 442 of the *FMR-1* gene plus five additional amino acids (Cys-Thr-His-His-Leu) as a result of the cloning procedure. Expression and purification of the fusion protein were as described⁹. Antibodies were affinity-purified using columns of GST and (GST)-*FMR-1* fusion protein successively. Pellets of lymphoblastoid cell lines were homogenized in a buffer containing 10 mM HEPES, 300 mM KCl, 100 μ M CaCl_2 , 5 mM MgCl_2 , 0.05% Tween and 0.45% Triton X100, pH 7.4. Cell homogenates were spun down for 10 min (10,000g). The supernatants were incubated with protein A-Sepharose (Pharmacia) for 2 h at 4°C to remove the IgG present in the lymphoblastoid cells. Protein A-Sepharose was spun down and the supernatant was incubated overnight with $\alpha 765$ and protein A-Sepharose at 4°C. After washing (3 $\times$) the immunoprecipitate, sample buffer was added and the precipitates were electrophoresed in SDS-polyacrylamide (8% polyacrylamide gel, 1% crosslinking) and electroblotted. Polypeptides were visualized using 1,000 $\times$ diluted $\alpha 765$ and a 1,000 $\times$ diluted alkaline phosphatase-conjugated goat anti-rabbit IgG as a second antibody. Polypeptides were detected using naphthol AS-MX phosphate and 4-aminodiphenylamine diazonium sulphate or AMPPD (TROPIX) as substrates.

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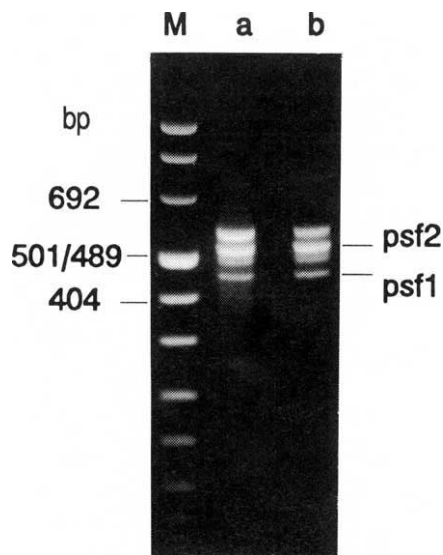


FIG. 2 Different splice products from the 3' end of the *FMR-1* gene visualized by reverse transcriptase PCR amplification of cDNA. Total RNA was isolated from leukocytes (lane a) and fetal brain (lane b)¹⁴. Alternative splicing involved exons 15 and 17 of *FMR-1* (D. L. Nelson, personal communication). The lengths of the PCR products were 563, 512, 488 and 437 bp. Length of DNA marker (lane M; factor VIII; Boehringer) bands are indicated in base pairs. The different splice variants shown were found to be present in isolated cDNAs (data not shown).

METHODS. Total RNA isolation was according to ref. 14. The LiCl method was used (procedure C) with several modifications. After overnight incubation in 3 M LiCl/6 M urea, samples were spun down for 20 min at 25,000 r.p.m. at 4°C and treated with proteinase K (10 µg ml⁻¹) for 30 min at 37°C before extraction with phenol/chloroform. Five µg RNA was reverse-transcribed as described in ref. 8, except that instead of precipitating the cDNA, 2 µl was directly used for PCR. PCR was done on 2 µl cDNA solution with the primer set K7 and K8. Primer K7: 5'-GCTAGTCTAGACCACCACCAAT-3' and primer K8: 5'-TTAGGGTACTCCATTACGAG-3' were derived from positions 1,462–1,485 and 1,954–1,974 of the published *FMR-1* cDNA sequence³. Amplification and analysis of PCR products was done as described¹⁵.

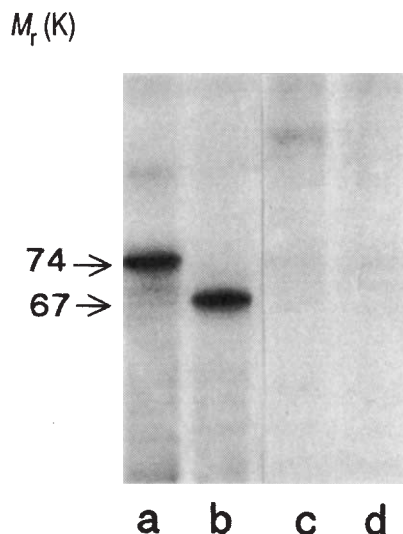


FIG. 3 Immunoprecipitation of COS-1 cells transfected with *FMR-1* (psf-2, lane a), or an alternative splice variant of *FMR-1* (psf-1, lane b). Immunoprecipitation of mock-transfected COS-1 cells or COS-1 cells transfected with *FMR-1* cloned in pSG5 (ref. 12) in the antisense direction are shown in lanes c and d, respectively. The molecular weight of the immunoprecipitated proteins is indicated on the left. COS-1 cells were labelled for 2 h with ³⁵S-methionine and ³H-leucine, 72 h after transfection. Cells were homogenized and used for immunoprecipitation with polyclonal antibody α765 raised against a fusion protein of glutathione S-transferase.

METHODS. *FMR-1* cDNA was cloned into the *Eco*RI site of the eukaryotic expression vector pSG5 (ref. 12) either as a 3,765-bp cDNA³ (psf-2) or as a 3,690-bp *FMR-1* splice variant missing in the 3' end of the 75-nucleotide coding sequence (1,598–1,672) but leaving the reading frame intact (psf-1). Transfections were achieved using the DEAE-dextran method¹⁶.

material in the lymphoblastoid cell lines from these patients is in agreement with the absence of *FMR-1* mRNA in these cells (ref. 8, and data not shown).

In the cell line of one patient, however, we found the same molecular species as in the controls (Fig. 1B). The patient has a mosaic DNA pattern, with one-third of the cells carrying a premutation and the rest a full mutation (data not shown). The CpG island preceding the CGG repeat is unmethylated in the premutation, in contrast to the full mutation. The premutation allele of the mosaic patients is expressed into *FMR-1* mRNA⁸ (data not shown), and subsequently translated into FMRPs (Fig. 1B, lane c).

The finding of four protein products rather than one was unexpected, and the possibility was envisaged that this could be due to alternative splicing. Figure 2 shows that there were several *FMR-1* mRNA species in lymphoblastoid cells and fetal brain. The set of primers used in the polymerase chain reaction (PCR) were chosen from near the 3' end of the open reading frame and amplified at least four splice products, and we have evidence for an even higher number of splice variants¹¹. Several of these splice variants have been found in isolated cDNAs.

Two of the *FMR-1* cDNA clones representing the alternative splice variants psf-2 and psf-1 (Fig. 2) were cloned in expression vector pSG5 (ref. 12) and these constructs were transiently expressed in COS-1 cells. Both the larger and the smaller

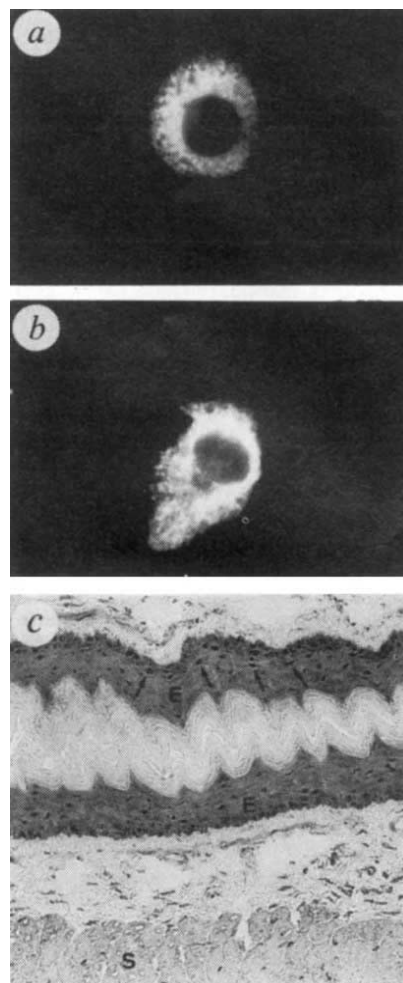
construct seemed to encode stable proteins that were recognized by antibodies α765 (Fig. 3, lanes a and b) and α1079 (data not shown). The estimated *M_s* were 74K and 67K for the proteins encoded by psf-2 and psf-1, respectively. No protein product was detected in mock-transfected COS-1 cells or in COS-1 cells transfected with *FMR-1* cDNA cloned in pSG5 in the antisense orientation (Fig. 3, lanes c and d).

The intracellular localization of the protein products of these different splice variants was investigated in COS-1 cells. It was shown by immunofluorescence microscopy using α765 antibodies that the protein encoded by psf-2 was to be found in the cytoplasm, despite the presence of a putative nuclear location signal (Fig. 4). The results were the same with the protein encoded by psf-1 and antibody α1079 raised against a synthetic peptide spanning the nuclear location signal. This observation was confirmed in mouse oesophagus, which is known to strongly express *FMR-1* (ref. 13). FMRP was seen in the cytoplasm of the keratinocytes throughout the thick stratified squamous epithelial lining, with no staining of the keratin layer (Fig. 4c). Some cells showed an intense nuclear labelling.

Until now there has been uncertainty as to whether the CGG repeat is translated into protein. Our experiments (Fig. 1) on the expression of the *FMR-1* gene in lymphoblastoid cell lines show that the protein products of the controls and the mosaic patient are of the same size. Mosaic patients have a full mutation (>200

FIG. 4 Cytoplasmic localization of FMRP in transfected COS-1 cells using an indirect immunocytochemical method¹⁷. The $\alpha 765$ antibodies were visualized using goat anti-rabbit immunoglobulins conjugated with fluorescein. *a*, COS-1 cells transfected with p ψ -1. *b*, COS-1 cells transfected with p ψ -2. As a control, non-transfected COS-1 cells were used. *c*, Cryostat section (6 μ m) of the oesophagus from an adult mouse incubated with polyclonal antibody $\alpha 765$, raised against fusion protein. Reaction product (dark grey staining) is seen in the cytoplasm of the cell, forming a thick stratified squamous epithelium, and not in the keratin layer. No product was seen using preimmune sera. Some cells show an intense labelling of the nucleus. E, stratified squamous epithelium; S, skeletal muscle; arrows indicate nuclei labelled for FMRP.

METHODS. For immunohistology cryostat sections were labelled with primary antibody and visualized with peroxidase-conjugated, swine-anti-rabbit immunoglobulins (DAKO, Denmark). Peroxidase was detected with 0.1% 3,3'-diaminobenzidine-HCl (Sigma) With 0.1% hydrogen peroxide. Sections were not counterstained.



are of the same size. Mosaic patients have a full mutation (>200 repeats) in a proportion of their cells and a premutation (50–200 repeats) in others. The premutation allele is transcribed, unlike in the full mutation⁸. In this patient the premutation allele contains 65 CGG copies more than the average control. If the CGG repeat were translated into a stretch of arginines, then protein products with an increased molecular mass would have been expected in the mosaic patient. As this was not found (Fig. 1*B*), it can be concluded that the translation of *FMR-1* starts distal to the CGG repeat. Expression of p ψ -1 and p ψ -2 in COS-1 cells showed that translation can start at an ATG initiation codon distal to the CGG repeat, as no ATG codon is present proximal to the CGG repeat in the open reading frame of the *FMR-1* gene (S. T. Warren, personal communication). Considering these data together, it is unlikely that the CGG repeat is part of the coding sequence.

In fragile X syndrome the amplification of the CGG repeat

blocks transcription of the *FMR-1* gene⁸ and this results in the absence of FMRP (Fig. 1) and causes mental retardation. The mosaic patient is mentally retarded despite the expression of *FMR-1* proteins. It is therefore assumed that the proportion of cells expressing FMRP is insufficient to maintain normal function. Alternatively, it could be that the mosaic pattern found in lymphoblastoid cell lines is not representative of the situation either in other tissues (like the brain) or during the developmental stages in which the FMRP expression is essential.

With respect to the further elucidation of the function of the FMRPs, it is important that the protein is predominantly present in the cytoplasm. Our results will make it possible to isolate the FMRPs, characterize them further, and study their putative differential function. This could provide insight into the mechanism leading to the manifestations of the fragile X syndrome. □

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Peptide binding inhibits protein aggregation of invariant-chain free class II dimers and promotes surface expression of occupied molecules

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EFFICIENT egress of major histocompatibility complex (MHC) class I molecules from the endoplasmic reticulum (ER) depends on peptide binding¹⁻⁷. For MHC class II molecules, invariant chain (Ii) promotes ER exit of newly assembled, peptide-free dimers⁸⁻¹⁴. This raises the question of whether a mechanism exists elsewhere in the cell that dictates selective expression of peptide-associated class II molecules. We report here that dissociation of MHC class II-Ii complexes at low pH and physiological temperature leads to inclusion of empty class II in protein aggregates, and that this aggregation is specifically prevented by peptide binding. Combined with data showing that antigen exposure increases cell surface class II expression on living cells by a post-translational mechanism¹², these results provide evidence for peptide-dependent intracellular editing of class II dimers, which limits surface expression of empty molecules unsuitable for antigen-specific T-cell activation.

To simulate *in vitro* the conditions present in the endosomal/lysosomal compartment(s) in which class II-peptide interaction appears to take place, spleen cell lysates containing pulse-labelled proteins were exposed to acidic pH at 37 °C, with or without added peptide antigen. After neutralization, class II molecules were immunoprecipitated and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) without sample heating. Compact dimers characteristic of the peptide loaded state¹⁵⁻¹⁷ were observed when an A α A β ^k-binding peptide (HEL 46-61) was included in low pH-treated lysates from H-2^k cells (Fig. 1a, upper panel). Stabilization was specific, as the HEL peptide did not generate SDS-stable dimers from E α E β ^k molecules in the same lysate, whereas an E α E β ^k binding peptide (MCC 88-103) showed the reciprocal effect (Fig. 1a, lower panel). Samples treated at low pH characteristically showed a high background and the removal of visible aggregates by centrifugation before immunoprecipitation eliminated the background (Fig. 1b). These cleaner autoradiographs showed that low pH exposure in the absence of specific peptide led to the loss of most (>80-90%) of the immunoprecipitable A α A β ^k molecules in these lysates (Fig. 1a,b, upper panels), despite the use of an antibody that reacts with all conformations of A α A β ^k (ref. 18). In contrast, a greater fraction of the labelled A α A β ^k (up to 60% in the best experiments) was recovered as stable dimers in the presence of specific peptide (Fig. 1a,b, upper panels).

Ii acts as a chaperone for class II in the ER^{11,13}. It inhibits peptide binding^{9,10,14} and is absent from compact class II dimers produced within living cells^{12,19,20}. The peptide-induced generation of compact dimers in lysates was prevented by precipitation of Ii, which removed >90% of the pulse-labelled class II molecules (Fig. 2a). As expected of molecules in the ER, the lysate-generated stable dimers were endoglycosidase-H sensitive, in contrast to those generated spontaneously by the cell (Fig. 2b). Although they derived from Ii-associated class II molecules, compact dimers generated *in vitro* could not be precipitated by anti-Ii antibodies (Fig. 2c), consistent with Ii

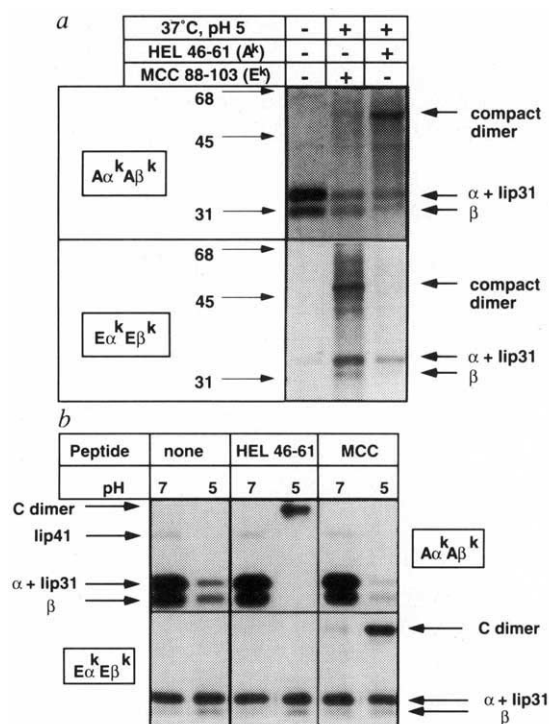


FIG. 1 Peptide effects on newly synthesized, Ii-associated MHC class II molecules exposed to low pH. **a**, SDS-PAGE of unheated anti-MHC class II immunoprecipitates from lysates of pulse-labelled H-2^k spleen cells. The lysates were treated before immunoprecipitation at 4 °C and neutral pH (lane 1), exposed to pH 5 at 37 °C in the presence of moth cytochrome c 88-103 peptide (lane 2), or exposed to pH 5 at 37 °C in the presence of HEL 46-61 peptide (lane 3). The upper panel shows anti-A^k immunoprecipitates, the lower panel shows anti-E^k precipitates. Arrows on the right indicate the location of separate MHC class II β-chains, superimposed, α- and Iip31 chains, and stable (compact) MHC class II αβ-dimers; arrows on the left show positions of M_r markers. **b**, Centrifugation removes aggregates formed during low pH treatment of cell lysates, emphasizing both the highly specific peptide-mediated conversion of unstable MHC class II complexes to stable dimers and the loss of immunoprecipitable A α A β ^k molecules on low pH treatment in the absence of peptide. Lysates from labelled H-2^k spleen cells treated at 37 °C at pH 7 or 5 in the presence or absence of the indicated peptides were spun in a microfuge before immunoprecipitation and gel analysis. Upper panel shows immunoprecipitation with anti-A^k, lower panel shows immunoprecipitation with anti-E^k.

METHODS. Spleen cells from CBA/J (H-2^k) mice were pulse-labelled for 20 min with ³H-leucine, lysed, and the lysate precleared as previously described¹². The lysate was then divided into aliquots in fresh microfuge tubes. Peptide (hen egg lysozyme (HEL) 46-61, single-letter amino-acid code NTDGSTDYGLQINSR or moth cytochrome c (MCC) 88-103, ANERADLIAYLKQATK) was added to 100 μM final concentration to appropriate aliquots, and the pH lowered to 5 by addition of a premeasured amount of 2 M acetic acid. Control lysate was incubated at 4 °C (**a**) or 37 °C (**b**) for 30 min without additions and at neutral pH; low pH-treated lysates were incubated at 37 °C for 30 min. After incubation, low pH samples were rapidly returned to neutral by addition of 2 M Tris, and all samples allowed to come to room temperature. Immunoprecipitation with Protein A-Sepharose-bound monoclonal antibody (10-2.16 (ref. 31) for A α A β ^k, 14.4.4S (ref. 32) for E α E β ^k), elution at room temperature in SDS sample buffer containing 2-mercaptoethanol, and SDS-PAGE done in the absence of sample heating were as previously described¹². In **b**, the lysates were centrifuged for 5 min at room temperature at 14,000 r.p.m. in an Eppendorf microfuge after control or low pH treatment and the supernatant used for immunoprecipitations.

being removed from $A\alpha^k A\beta^k$ by the low pH exposure at 37 °C, permitting stable peptide binding. Western blotting revealed that in lysates prepared at 4 °C and neutral pH, some mature, post-Golgi class II was already in an aggregated or spontaneously aggregating form (Fig. 2d, labelled α''). Consistent with the post-translational processing of this class II, intact Ii was not present in such spontaneous aggregates. Conversely, most of the

Ii and newly synthesized (pre-medial Golgi) class II molecules originally present in the lysate (labelled α and α') were absent from such spontaneous aggregates. These latter molecules were found, however, in the sedimentable material generated by low pH treatment in the absence of peptide, irrespective of whether centrifuged before or after neutralization. As with the metabolically labelled samples, a greater proportion of the immature

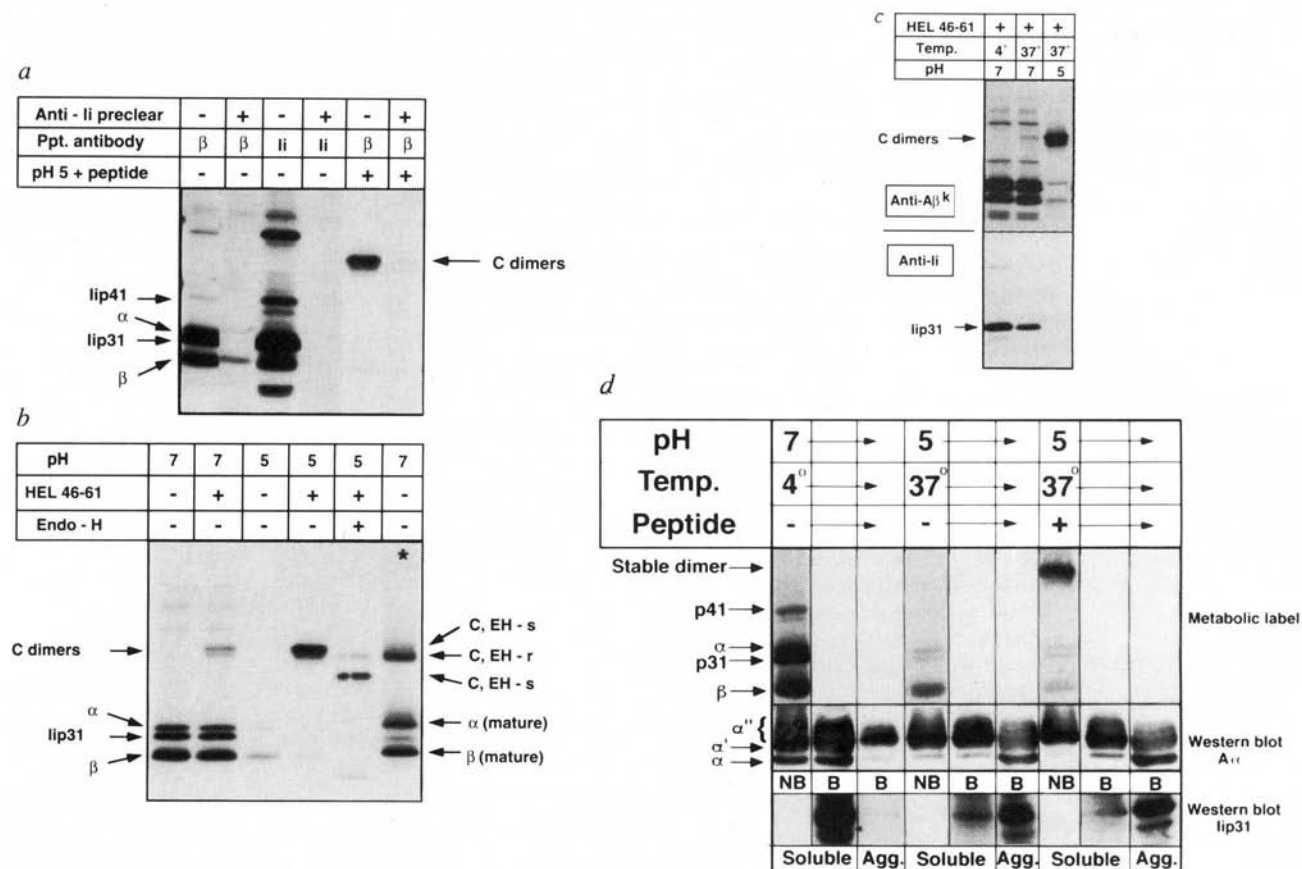


FIG. 2 Relationship of Ii-association and post-translational maturation to MHC-class II low pH-induced aggregation and stable dimer formation. **a**, Stable MHC class II dimers produced by exposure of newly synthesized class II molecules to low pH and peptide derive from Ii- $\alpha\beta$ complexes. MHC class II molecules associated with Ii were depleted by immunoprecipitation with anti-Ii before lysate treatment. Lane 2 shows the removal of ~90% of the $A\alpha^k A\beta^k$ molecules by this procedure; lane 4 shows the complete absence of residual Ii. Lane 5 shows the production of stable dimers by low pH and peptide using intact lysate; lane 6 shows the absence of such dimers using lysate depleted of Ii-associated MHC class II. **b**, Endoglycosidase-H sensitivity of stable dimers formed from newly synthesized MHC class II molecules by exposure to peptide at low pH. Lysates of labelled H-2^k spleen cells were treated as indicated above each lane, then analysed either with or without treatment with endoglycosidase-H (Endo-H) before SDS-PAGE. The rightmost lane indicated by an asterisk shows the migration of labelled class II molecules after 4 h chase, when virtually all glycans have achieved endo-H resistance. **c**, Ii is not detected associated with stable MHC class II dimers produced by low pH and peptide exposure. The upper panel shows immunoprecipitations using anti- $A\beta^k$ antibody; the lower panel shows immunoprecipitations using antibody to the cytoplasmic tail of Ii. **d**, Presence of mature class II but not Ii in aggregates from untreated cell lysates, the appearance of both Ii and newly synthesized class II in aggregated material after low pH exposure at 37 °C, and the selective partial exclusion of class II dimers but not Ii from aggregates by peptide exposure during low pH treatment. **METHODS**. CBA/J spleen cells were labelled, lysed, and treated as indicated above each gel lane, using the procedures described in the legend to Fig. 1b, except that in **a**, one portion of the labelled cell lysate was depleted of Ii and Ii-associated MHC class II before low pH

and peptide treatment by two sequential exposures to a combination of the anti-mouse Ii antibodies In-1 (ref. 33) and P4H5 (ref. 34) and Pansorbin, followed by exposure to Protein A-Sepharose alone. $A\alpha^k A\beta^k$ was immunoprecipitated using 10-2.16; Ii was precipitated using In-1, directed against an epitope in the cytoplasmic tail of Ii present on both intact and partially cleaved fragments of mouse Ii associated with MHC class II in the endocytic pathway. **b**, CBA/J spleen cells were pulse-labelled, lysed, and the lysates treated according to the methods for Fig. 1b and as indicated for each lane of the gel. Samples were then analysed by SDS-PAGE without sample heating after mock or actual treatment of the precipitated proteins with endoglycosidase-H, as previously described¹². The migration positions of stable dimers with endo-H sensitive (C,EH-s) and endo-H resistant (C,EH-r) glycans are indicated. The upper label (C,EH-s) refers to the position of the sensitive dimers before endo-H treatment, the lower label to such sensitive dimers after treatment. **c**, $A\alpha^k A\beta^k$ was precipitated using 10-2.16, Ii using In-1. **d**, The upper panels show the results of immunoprecipitation studies of metabolically labelled $A\alpha^k A\beta^k$ from lysates treated as indicated in each lane; the lower panels show the results of western blots of cold lysates from spleen cell preparations treated in the same manner as the labelled cell lysates, then developed after blotting with either In-1 monoclonal antibody for Ii or a rabbit antiserum to the C-terminal cytoplasmic segment of $A\alpha^k$. The bands shown represent either the signal from the p31 form of Ii or the various glycosylated forms of $A\alpha$. α , completely endo-H sensitive form; α' , partially endo-H-sensitive form; α'' , fully endo-H-sensitive form. Almost all the α and α' forms are associated with Ii in spleen cells, whereas most α'' is in class II from which Ii has been removed. B and NB refer to whether the sample in the lane was heated before SDS-PAGE.

$A\alpha^k A\beta^k$, but not Ii, remained in the detergent-soluble fraction in the presence of specific peptide.

Low pH treatment without added peptide did not cause a similar loss of immunoprecipitable $E\alpha^k E\beta^k$; this correlated with a failure of low pH alone to remove most of the Ii from these dimers (Fig. 1a,b, lower panels). This behaviour of $E\alpha^k E\beta^k$ is more typical, as proteolysis is usually needed for removal of Ii^{14,21}. Exposure of lysates to peptides at 37 °C and neutral pH led to little or no generation of stable dimers or loss of immunoprecipitable $A\alpha^k A\beta^k$, which maintained its association with Ii under these conditions (Fig. 1a,b, upper panels). This indicates that dissociation of class II molecules from Ii precedes inclusion of class II into the low pH-induced aggregates. Thus, peptide binding to $A\alpha^k A\beta^k$ not only induces a structural change in the class II dimer leading to resistance to SDS denaturation, it also selectively rescues dimers lacking associated Ii from acid- and temperature-induced aggregation with other proteins.

Antigen (HEL) can generate compact dimers from $A\alpha^k A\beta^k$ molecules that would otherwise be lost to immunochemical detection within 2–4 h of synthesis in mouse spleen cells and substantially increases surface class II expression¹². This *ex vivo* phenomenon parallels the present results and also the ability of peptide to reverse aggregation of purified Ii- and peptide-free human class II molecules¹⁶. Together with our observations here of aggregated, mature, Ii-free class II in fresh spleen cell lysates and the selective exclusion of Ii-free, peptide-associated class II from aggregates generated at low pH *in vitro*, these findings suggest that cells 'edit' MHC class II molecules to achieve selective expression of peptide-loaded molecules on the cell surface. This editing appears to result from the aggregation

(homotypic or heterotypic) in a low pH environment of Ii-free class II molecules that have failed to capture the peptide ligand necessary for adopting a denaturation/aggregation-resistant conformation. The similarity between the conditions required for class II aggregation *in vitro* and the characteristics of endocytic compartments in which Ii is removed before peptide binding to class II in living cells suggest that aggregation could prevent exit of class II from late endocytic/prelysosomal compartments and may ultimately result in class II degradation. But analysis of class II from isolated endocytic vesicles will be necessary to establish this point directly. Class II molecules successfully acquiring peptide in the low pH environment would presumably avoid this fate and be routed to the cell surface. Escape of empty class II molecules to the plasma membrane by avoidance of the acidic editing compartment could explain the enhanced capacity of class II molecules expressed by Ii-negative cells to present antigenic peptides^{22–24}.

We¹² and others²⁵ have described a significant cohort of surface class II molecules that because of their sensitivity to SDS dissociation were suggested to have unoccupied binding sites. The present findings are inconsistent with this interpretation and imply that these might be dimers that have adopted a peptide-dependent conformation needed to avoid aggregation/degradation, without acquiring the SDS-stable phenotype. Figure 3 shows that SDS-sensitive $A\alpha^k A\beta^k$ dimers surviving for several hours after synthesis could not be converted to the SDS-resistant phenotype upon low pH treatment in the presence of HEL 46-61, despite the absence of Ii. Similarly, newly synthesized $A\alpha^d A\beta^d$ molecules treated at low pH in the presence of a known $A\alpha^d A\beta^d$ -binding peptide (haemagglutinin

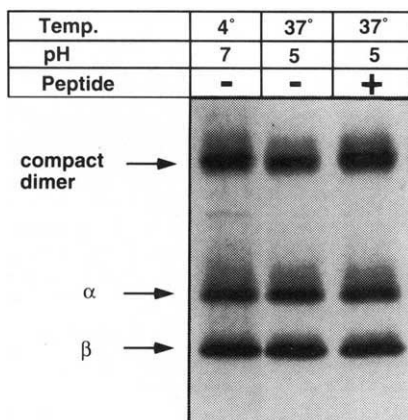


FIG. 3 Low pH exposure in the presence of peptide does not convert post-Golgi, unstable MHC class II molecules to stable dimers. H-2^k spleen cells were pulse-labelled, then chased for 4 h before lysis, immunoprecipitation of any remaining Ii-associated class II molecules, treatment of Ii-cleared lysates with peptide and low pH, and immunoprecipitation with anti- $A\beta^k$ antibody. Lysate treatments are indicated above each lane.

METHODS. CBA/J spleen cells were pulse-labelled, then subjected to a 4-h chase. After detergent lysis, all Ii and Ii-associated class II molecules were removed by several sequential precipitations using In-1 and P4H5 anti-Ii antibodies. The remaining material was treated as indicated, the $A\alpha^k A\beta^k$ molecules precipitated using 10-2.16 antibody, and the precipitated proteins analysed by SDS-PAGE without sample heating.

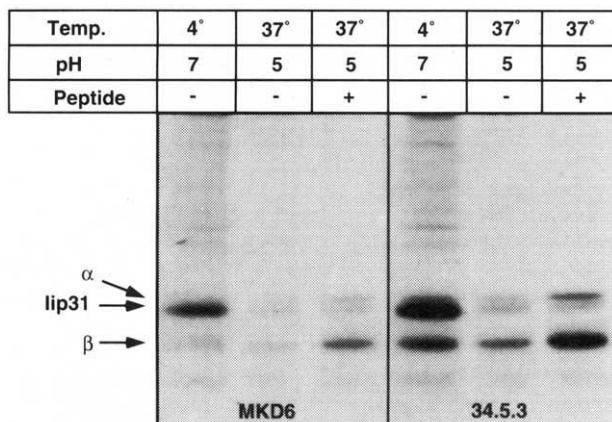


FIG. 4 Peptide binding can prevent low pH-induced aggregation of Ii-free class II dimers without inducing stability as assayed by SDS-PAGE. H-2^d spleen cells were pulse-labelled, lysed, and the lysates treated as indicated above each lane of the gel. Immunoprecipitations were done from treated lysates with either MKD6 (ref. 36) (anti- $A\beta^d$) monoclonal antibody (left lanes) or 34.5.3 (ref. 37) (anti- $A\beta^{b,d}$) (right lanes).

METHODS. BALB/c (H-2^d) spleen cells were pulse-labelled, lysed, and the lysates treated as described in the legend to Fig. 1, except that the peptide used was HA 130-142 (HNTNGVTAACSHE), known to bind well to $A\alpha^d A\beta^d$ class II molecules²⁶. $A\alpha^d A\beta^d$ was precipitated from the treated lysates using either MKD6 or 34.5.3 antibodies and analysed by SDS-PAGE without sample heating.

130-142)²⁶ showed increased antibody immunoprecipitability compared with A α A β ^d exposed to low pH without specific peptide, but the additional dimers observed in the presence of peptide did not acquire resistance to SDS-induced dissociation. It thus seems that long-lived, SDS-sensitive class II dimers detected in pulse-chase experiments or on the cell surface are largely peptide-associated rather than empty, a conclusion supported in ref. 27. Therefore, although stable compact dimers are clearly created by peptide loading and although newly synthesized, empty dimers are unstable, not all SDS-unstable dimers are necessarily peptide-free. These latter conclusions may be of relevance in interpreting the behaviour of class II molecules from cells with defects in the class II processing pathway^{25,28,29}. Class II molecules of distinct isotype, allele, and species origin differ markedly in the proportion of stable and unstable dimers generated late after synthesis^{12,30}; this may reflect the effects of the different cohorts of peptide they capture, variations in intrinsic stability of the $\alpha\beta$ dimer, or both.

Because T cells recognize antigen as peptides bound to MHC molecules on the cell membrane, empty MHC molecules represent a distraction for the TCR and CD4 or CD8 coreceptors during the molecular scanning process which leads to effective TCR engagement and T-cell activation. The present data, together with results previously reported for the MHC class I pathway¹⁻⁷, make it apparent that MHC proteins have structural properties that permit efficient intracellular discrimination between peptide-loaded and empty molecules, reducing to a minimum the irrelevant MHC molecules that reach the cell surface. □

HIV induces thymus depletion *in vivo*

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HUMAN immunodeficiency virus (HIV) disease is typified by declining CD4⁺ T lymphocyte counts in the peripheral circulation, a loss which may be secondary to accelerated destruction, to suppressed differentiation, and/or to sequestration of circulating cells into tissue spaces. As it is hard to distinguish between these possibilities in human subjects, the pathogenic mechanisms associated with HIV infection are unclear. In particular, little is known about the events that occur within infected lymphoid organs in which most CD4 T lymphocytes mature and function^{1,2}. To obtain a better description of HIV pathogenesis *in vivo*, we have implanted human haematolymphoid organs into the immunodeficient SCID mouse to create the SCID-hu mouse^{3,4}. We have previously shown that these organ systems promote long-term multilineage human haematopoiesis and are permissive for infection with HIV^{5,6}. Here we report that human thymopoiesis is suppressed by HIV infection, thereby precluding regeneration of the peripheral T-cell compartment.

SCID-hu mice were constructed with conjoint organ implants of human fetal liver and thymus, providing both human haematopoietic progenitor cells and the stromal microenvironment in which T-cell maturation occurs. Such Thy/Liv SCID-hu mice support long-term, multilineage human haematopoiesis, including differentiation of phenotypically normal and functionally competent human T cells^{5,7,8}. After infection with the primary HIV-1 isolate (SM), viral replication was detected at varying times postinfection by an enzyme-linked immunosorbent assay (ELISA) for HIV-1 (Fig. 1a). The p24 level peaked to a mean value of 348 pg per 10⁶ cells by day 23. At days 28 and 34 postinfection, mean values decreased to 177 pg and 93 pg per 10⁶ cells, respectively. This fall in levels of viral replication may have reflected changes in the state of viral activation and/or destruction of infected cells.

Immunohistological analysis of Thy/Liv implants showed signs of viral replication and progressive loss of CD4⁺ cells. There were only rare foci of infected cells one week after HIV(SM) inoculation (Fig. 1b), but by 4 weeks infected cells were seen throughout the thymus, particularly in the cortex (Fig. 1c). These cells included small round cells as well as cells with a dendritic morphology (Fig. 1d). During the same period, the distribution of CD4⁺ and CD8⁺ thymocytes in the Thy/Liv parenchyma was altered significantly. In contrast to the normal pattern of dense staining in cortical areas and more diffuse staining in the medulla (Fig. 1e), HIV(SM)-infected thymuses showed cortical regions that were virtually devoid of thymocytes expressing CD4 and/or CD8, and medullary regions that lacked cells expressing CD4 (Fig. 1f and g).

Subpopulations of thymocytes in the HIV-infected Thy/Liv implant were analysed by flow cytometry. Most thymocytes from uninfected control SCID-hu mice as well as animals injected with heat-inactivated virus expressed both the CD4 and CD8 surface markers, and thymocytes expressing either CD4 or CD8 were present at a ratio of ~2.5:1 (Fig. 2a and b). In contrast, when mice were infected with HIV(SM), the CD4⁺8⁺ compartment was eliminated and the CD4:CD8 ratio was inverted by 5 weeks postinoculation (Fig. 2c and d). Similar results were obtained when human Thy/Liv implants were infected with other primary isolates of HIV-1. Infection with HIV(TY) and HIV(EW) gave a pronounced decrease in the percentage of CD4⁺CD8⁺ thymo-

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cytes over time and an inversion of the CD4 : CD8 ratio (Table 1). When infected with JR-CSF, an infectious molecular clone of a primary HIV-1 isolate⁹, thymocyte profiles changed with a similar trend but with delayed kinetics (Table 1).

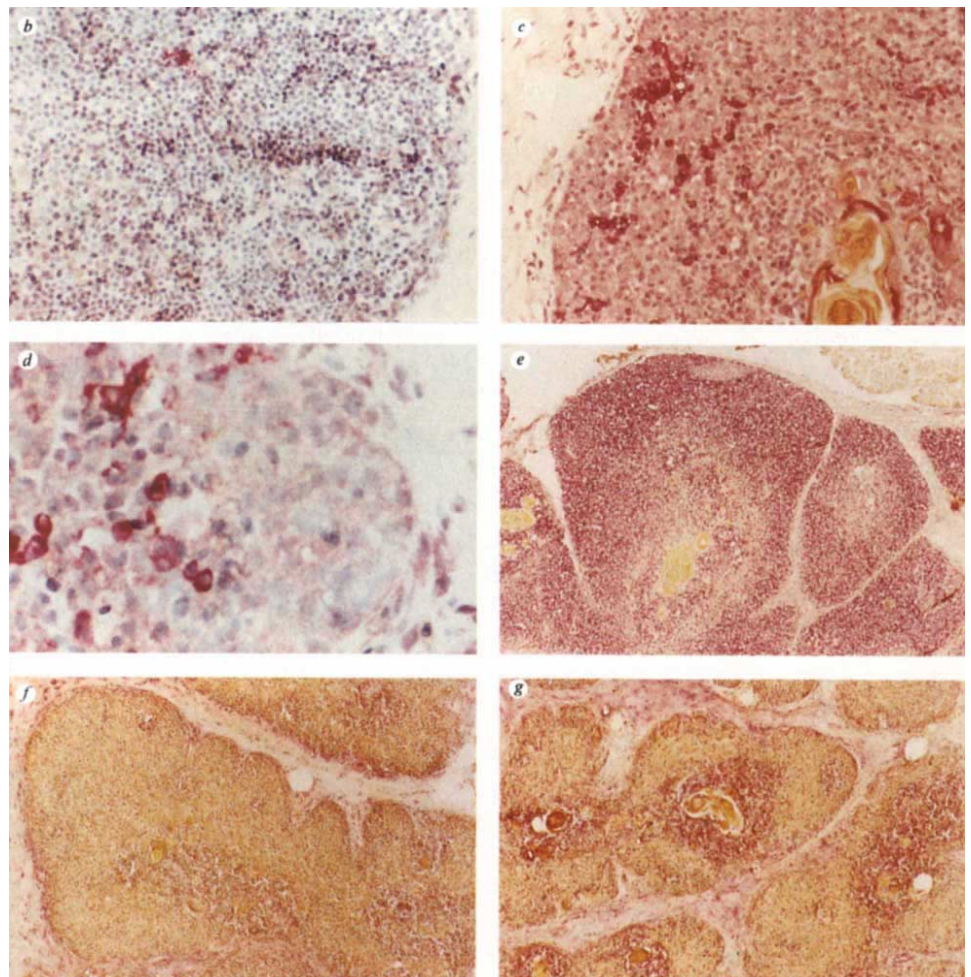
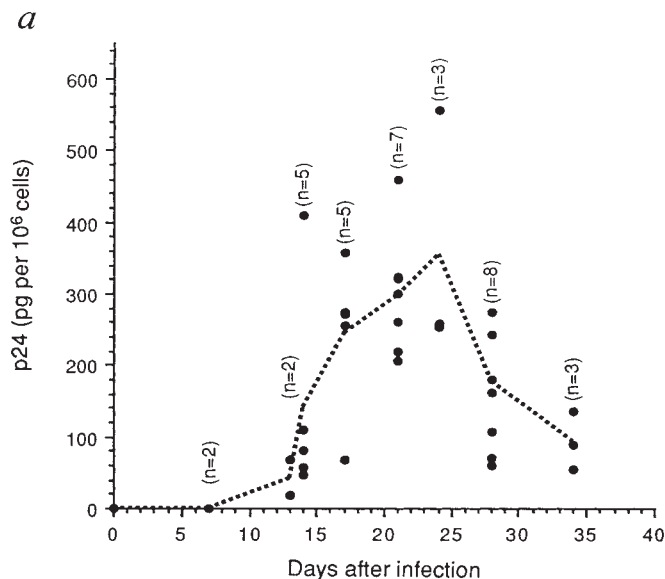
The decreased representation of surface CD4⁺ thymocytes following infection was probably not due to downregulation of CD4 expression. By multiparameter flow cytometry, all of the single-positive CD8⁺ cells were high in CD3 and most of the

CD4⁺CD8⁺ cells were CD3-negative (data not shown). These patterns are inconsistent with CD4 downregulation in the CD4⁺CD8⁺ and CD4⁺ populations, respectively. Rather, overall thymus cellularity was reduced (Table 1) and there was an accumulation of cells with increased side scatter and reduced forward scatter profiles (Fig. 2e and 2f), typical of dead or dying lymphocytes^{10,11}.

Microscopy was used to evaluate the morphological correlates

FIG. 1 Viral antigen expression pattern over time (a–d) and alterations in thymus composition (e–g) following infection with HIV(SM). a, At various times after infection with HIV(SM), thymocyte suspensions from Thy/Liv grafts in SCID-hu mice were assayed by ELISA for p24 expression. b–d, Immunohistochemical staining of viral antigens in HIV(SM)-infected SCID-hu Thy/Liv implants at 1 week postinfection (b) ($\times 124$), and at 4 weeks postinfection (c–d) ($\times 124$ and $\times 309$, respectively). e–f, Immunohistochemical staining for CD4 expression in an uninfected Thy/Liv implant (e) ($\times 25$) and in an HIV(SM)-infected Thy/Liv implant at 4 weeks postinfection (f) ($\times 41$). g, Immunohistochemical staining for CD8 expression in an HIV(SM)-infected Thy/Liv implant at 4 weeks postinfection ($\times 41$).

METHODS. SCID-hu mice with human Thy/Liv implants were prepared as described⁵ and used within 3–11 months. In most experiments, control and experimental animals carried Thy/Liv grafts obtained from the same donor and engrafted on the same day. Before use, grafts were inspected and animals with viable grafts selected. The animals were infected by direct intrathymic injection⁷ with 10^3 TCID₅₀ HIV(SM) in a volume of ~ 25 μ l. Animals were killed at various times by cervical dislocation; cell suspensions were then prepared from implants for p24 analysis. Cells were solubilized in PBS containing 1% Triton X-100 and P24 assayed using a p24 ELISA kit (Dupont). Levels of p24 from individual animals are represented as dots; means at a given time are connected by the dashed line. For immunohistochemistry, grafts from normal and infected SCID-hu Thy/Liv mice were fixed in PBS/4% paraformaldehyde, frozen, sectioned and subsequently stained with either the anti-HIV antisera, GB (ref. 7), mAb OKT4 (ATCC) or mAb OKT8 (ATCC) as described⁴.



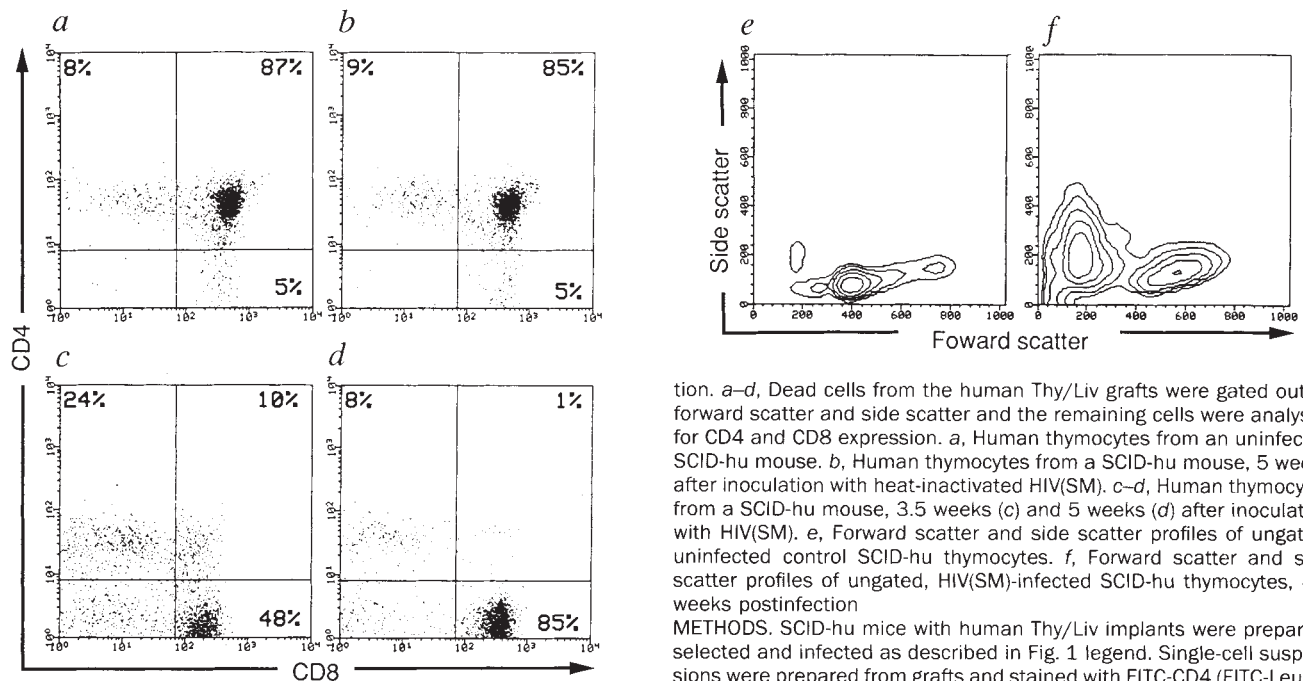


FIG. 2 Flow cytometric analysis of time-dependent loss of CD4-expressing cells and increased percentage of dead or dying cells as determined by forward scatter/side scatter profiles following HIV infec-

tion. *a-d*, Dead cells from the human Thy/Liv grafts were gated out by forward scatter and side scatter and the remaining cells were analysed for CD4 and CD8 expression. *a*, Human thymocytes from an uninfected SCID-hu mouse. *b*, Human thymocytes from a SCID-hu mouse, 5 weeks after inoculation with heat-inactivated HIV(SM). *c-d*, Human thymocytes from a SCID-hu mouse, 3.5 weeks (*c*) and 5 weeks (*d*) after inoculation with HIV(SM). *e*, Forward scatter and side scatter profiles of ungated, uninfected control SCID-hu thymocytes. *f*, Forward scatter and side scatter profiles of ungated, HIV(SM)-infected SCID-hu thymocytes, 4.5 weeks postinfection

METHODS. SCID-hu mice with human Thy/Liv implants were prepared, selected and infected as described in Fig. 1 legend. Single-cell suspensions were prepared from grafts and stained with FITC-CD4 (FITC-Leu3a; Becton Dickinson Immunocytometry Systems) and PE-CD8 (OKT8; ATTC) in PBS/2% FCS. Cells were washed, resuspended in PBS containing 1% paraformaldehyde and analysed with a single laser FACScan (Becton Dickinson Immunocytometry Systems).

of cell death in HIV-infected Thy/Liv implants more closely. In the light microscope, uninfected implants showed densely packed cortical thymocytes with non-condensed chromatin (Fig. 3*a*). Two weeks after HIV(SM) infection, cortical areas instead contained single cells or clusters of cells with highly condensed nuclear material (Fig. 3*b*). By electron microscopy, cortical thymocytes with varying stages of nuclear condensation were seen in close proximity to thymocytes with a regular pattern of heteromarginated chromatin (Fig. 3*c*). These observations are similar to those obtained when thymocyte destruction is induced

by glucocorticoids, anti-CD3 antibodies, or staphylococcal enterotoxin B in rodent thymuses *in vivo* or *in vitro*¹²⁻¹⁵. They suggest that HIV-induced cell death in the thymus occurs by the process of programmed cell death.

It has been shown that HIV infection may initiate programmed cell death *in vivo* and *in vitro*¹⁶⁻¹⁸, so we investigated whether this is associated with thymocyte depletion in the HIV-infected SCID-hu mouse. Ethidium bromide staining of cells from infected implants did not generate the DNA fragmentation ladder often, but not always, seen in cases of programmed cell

TABLE 1 Expression of CD4 and CD8 and number of cells per graft after infection with HIV primary isolates SM, EW, TY and with JR-CSF

	Control	SM	SM	TY	TY	EW	EW	JR-CSF	JR-CSF
Weeks postinfection		3-4	4.5-6	3-4	4.5-6	3-4	4.5-6	4-6	8
Number of mice	42	37	20	3	4	3	5	4	5
CD4 ⁺ 8 ⁺ (mean %)	78.9	29.7*	3.7*	29.3*	34.2*	51.0*	22.8*	75.5	48.6*
Range (%)	58-88	0.3-81	0.2-23	2-71	4-70	36-80	9-45	69-85	20-75
Standard error (%)	1.1	4.3	1.3	22.1	16.6	14.5	6.9	3.5	9.0
CD4 ⁺ 8 ⁻ (mean %)	14.3	16.7	9.0*	12.0	8.5	11.7	13.0	14.5	26.6*
Range (%)	7-35	1.3-30	1.5-22	5-17	1.9-16	11-12	3-36	10-18	15-36
Standard error (%)	0.8	1.2	1.4	3.6	2.9	0.3	5.3	1.8	3.7
CD4 ⁻ 8 ⁺ (mean %)	5.5	43.4*	76.5*	43.3*	55.2*	33.7*	55.2*	8.2	18.4*
Range (%)	3-13	7-89	50-92	8-65	12-88	7-48	18-87	4-11	8-32
Standard error (%)	0.3	4.0	2.9	17.8	18.5	13.3	12.1	1.5	3.9
Cells per graft (mean) ($\times 10^6$ cells)	183.8	33.7*	7.0*	27.5	101.2	50.5	10.5	220.0	37.4
Standard error ($\times 10^6$ cells)	33.0	12.3	1.7	23.8	86.4	26.5	5.2	92.1	12.3

SM and EW were isolated from patients classified as having symptomatic HIV infection and who were not on any antiviral drug regimen. The SM inoculum was a third passage stock which did not induce syncytia in culture yet was cytopathic for PHA blasts. EW was a second passage stock that was cytopathic for PHA blasts and which caused formation of syncytia. TY was isolated from a patient classified as having AIDS and who had briefly been on AZT treatment before switching to ddI. The TY inoculum was a second passage stock that showed no cytopathicity for PHA blasts. JR-CSF was prepared as described⁹.

* Comparisons that are significantly different from controls ($P \leq 0.001$ as determined by the StatView II unpaired two-tail t-Test analysis program).

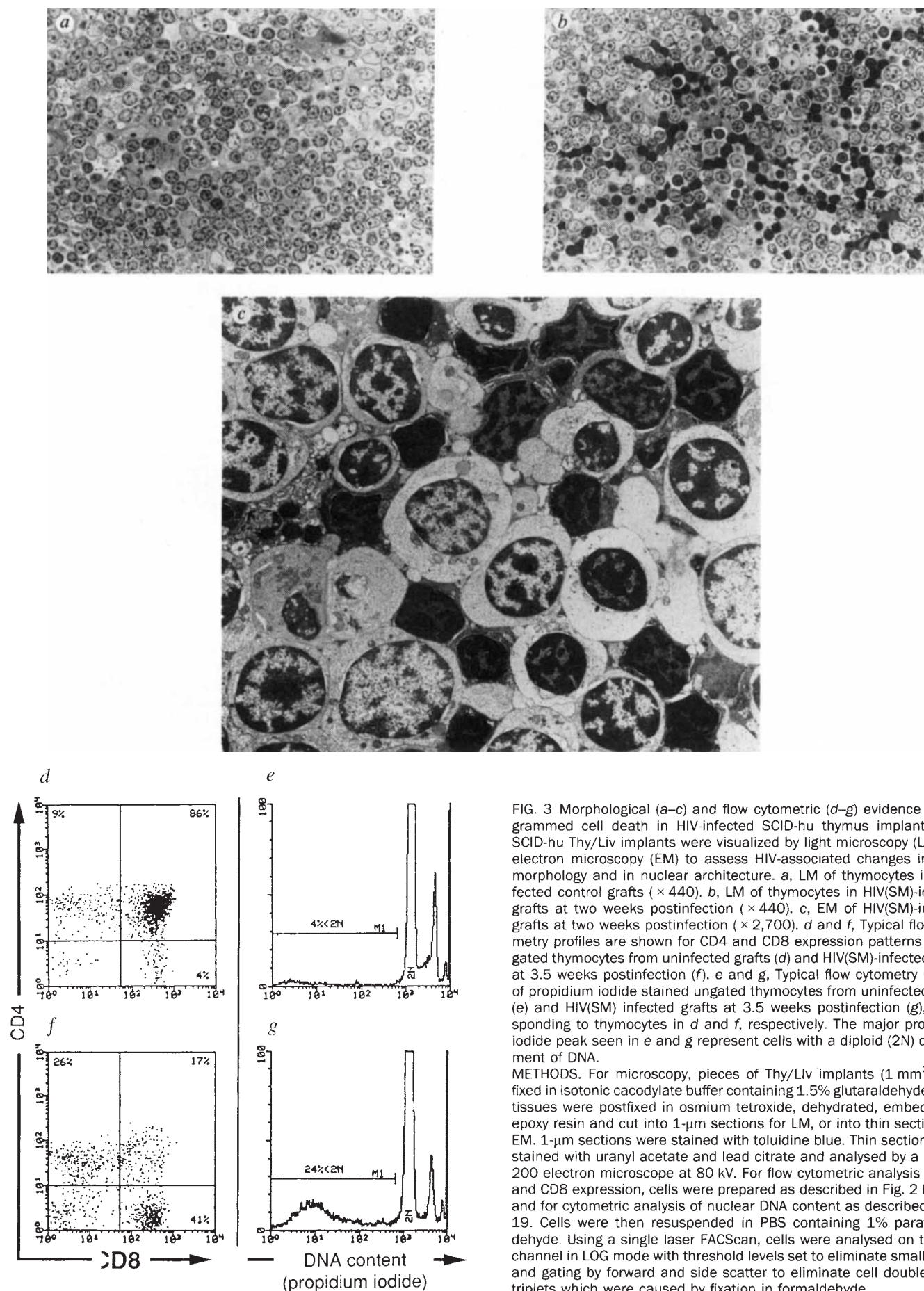


FIG. 3 Morphological (a–c) and flow cytometric (d–g) evidence of programmed cell death in HIV-infected SCID-hu thymus implants. a–c, SCID-hu Thy/Liv implants were visualized by light microscopy (LM) and electron microscopy (EM) to assess HIV-associated changes in gross morphology and in nuclear architecture. a, LM of thymocytes in uninfected control grafts ($\times 440$). b, LM of thymocytes in HIV(SM)-infected grafts at two weeks postinfection ($\times 440$). c, EM of HIV(SM)-infected grafts at two weeks postinfection ($\times 2,700$). d and f, Typical flow cytometry profiles are shown for CD4 and CD8 expression patterns on live gated thymocytes from uninfected grafts (d) and HIV(SM)-infected grafts at 3.5 weeks postinfection (f). e and g, Typical flow cytometry profiles of propidium iodide stained ungated thymocytes from uninfected grafts (e) and HIV(SM) infected grafts at 3.5 weeks postinfection (g), corresponding to thymocytes in d and f, respectively. The major propidium iodide peak seen in e and g represent cells with a diploid (2N) complement of DNA.

METHODS. For microscopy, pieces of Thy/Liv implants (1 mm^2) were fixed in isotonic cacodylate buffer containing 1.5% glutaraldehyde. Fixed tissues were postfixed in osmium tetroxide, dehydrated, embedded in epoxy resin and cut into $1\text{-}\mu\text{m}$ sections for LM, or into thin sections for EM. $1\text{-}\mu\text{m}$ sections were stained with toluidine blue. Thin sections were stained with uranyl acetate and lead citrate and analysed by a Phillips 200 electron microscope at 80 kV. For flow cytometric analysis of CD4 and CD8 expression, cells were prepared as described in Fig. 2 legend, and for cytometric analysis of nuclear DNA content as described in ref. 19. Cells were then resuspended in PBS containing 1% paraformaldehyde. Using a single laser FACScan, cells were analysed on the FL2 channel in LOG mode with threshold levels set to eliminate small debris and gating by forward and side scatter to eliminate cell doublets and triplets which were caused by fixation in formaldehyde.

death^{10,13,19-21} The DNA content of thymocytes from HIV(SM)-infected Thy/Liv implants was analysed using propidium iodide staining of permeabilized cells¹⁹. As a function of time after infection, flow cytometric analysis of such cells indicated a 6-fold rise in the percentage of cells with decreased DNA content over that of control thymocytes (Fig. 3d-g). Cells containing reduced amounts of DNA correlated exactly with cell populations that gave increased side scatter and reduced forward scatter flow cytometry profiles (data not shown). These patterns are consistent with the endonucleolytic cleavage observed during programmed cell death^{10,19,22}.

In summary, HIV-1 infection of Thy/Liv implants in SCID-hu mice is associated with a rapid depletion of CD4⁺CD8⁺ thymocytes and an inversion of the CD4 : CD8 ratio. The loss of CD4⁺ cells appears to be mediated at least in part by programmed cell death, an otherwise physiological function of the thymus. These events are relatively specific for HIV and do not occur when, for instance, the SCID-hu Thy/Liv system is infected with human cytomegalovirus²³. Given the many differences that exist between HIV infection in the SCID-hu system and in humans, it is still not clear how relevant our observations are to pathophysiological mechanisms of HIV. Cortical thymocyte depletion accompanied by compaction of the thymic epithelium has been noted, however, in fetuses aborted from HIV-1 seropositive women²⁴, in thymic biopsies from HIV-infected children²⁵, and in rhesus monkeys infected with simian immunodeficiency virus²⁶.

Several hypotheses about HIV-mediated T-cell depletion may be tested in the SCID-hu system. First, viral infection may kill thymocytes or their progenitors, either directly or indirectly. Second, HIV infection of thymic macrophages, dendritic cells, and/or epithelial cells²⁶⁻²⁸ may compromise their functions in regulating thymopoiesis^{29,30}. Finally, HIV infection of thymic stromal elements may facilitate pathologically the process of programmed cell death that normally occurs in the thymus. The SCID-hu mouse model may provide valuable insights into the mechanisms associated with HIV-induced thymocyte depletion and other aspects of HIV pathogenesis *in vivo*. Given the dominant role of the thymus *in utero* and in early childhood, these studies may be particularly relevant to the evaluation of paediatric AIDS. Moreover, as the thymus probably acts as a site of T-cell differentiation and maturation throughout life³¹, abrogation of thymopoiesis may represent a key pathogenic mechanism in all HIV-infected patients. □

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The SCID-hu mouse as a model for HIV-1 infection

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DURING normal fetal ontogeny, one of the first organs to harbour CD4-positive cells is the thymus¹. This organ could therefore be one of the earliest targets infected by human immunodeficiency virus type 1 (HIV-1) *in utero*. HIV-1-infected cells and pathological abnormalities of the thymus have been seen in HIV-1-infected adults and children, and in some fetuses aborted from infected women²⁻⁵. Studies of HIV-1 pathogenesis have been hampered by lack of a suitable animal model system. Here we use the SCID-hu mouse⁶ as a model to investigate the effect of virus infection on human tissue. The mouse is homozygous for the severe combined immunodeficiency (SCID) defect^{7,8}. The model is constructed by implanting human fetal liver and thymus under the mouse kidney capsule. A conjoint human organ develops, which allows normal maturation of human thymocytes. After direct inoculation of HIV-1 into these implants, we observed severe depletion of human CD4-bearing cells within a few weeks of infection. This correlated with increasing virus load in the implants. Thus the SCID-hu mouse may be a useful *in vivo* system for the study of HIV-1-induced pathology.

To determine the effect of HIV-1 infection on the human thymus, mice homozygous for the SCID genetic defect^{7,8} were transplanted with human fetal haematopoietic tissues. Fetal human thymus/liver (Thy/Liv) implants from 12 SCID-hu mice were either left unmanipulated or directly injected⁹ with heat-inactivated HIV-1 or with 1,000 infectious units (IU) of either the HIV-1_{JR-CSF} strain¹⁰ or a pool of five primary paediatric virus isolates. Thymocyte subset distribution 2.5 weeks post-inoculation was analysed using three-colour flow cytometry (Fig. 1 and Table 1).

Control Thy/Liv implants gave subset distribution patterns similar to those of normal human thymus: about 80 per cent of the cells stained for both the CD4 and CD8 markers, with most of the remaining cells staining as CD4-positive/CD8-negative. In contrast, all implants receiving the pooled paediatric virus inoculum were markedly depleted of the CD4/CD8 double-positive thymocyte population, as well as the CD4⁺/CD8⁻ cells.

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The majority of the remaining thymocytes were either CD3⁺/CD8⁺ or negative for both CD4 and CD8. One of the animals infected with the HIV-1_{JR-CSF} isolate was similarly depleted (Table 1). Implants that received the pooled virus inoculum were also severely depleted of mature CD4⁺/CD8⁺ cells. Thus HIV-1 infection *in vivo* results in depletion of both mature and immature thymocytes bearing CD4. This depletion is probably the result of death of thymocytes, although downregulation of CD4 could account for some loss of CD4⁺ cells. Infection and death of the CD4⁺/CD8⁺ precursor cells could account for the eventual loss of CD4⁺/CD8⁺ cells. This effect is specific for HIV-1, as infection of Thy/Liv implants with human cytomegalovirus does not result in thymocyte depletion¹¹.

To characterize further the effect of HIV-1 on Thy/Liv implants, a second series of animals was inoculated with 200 IU of either HIV-1_{JR-CSF} or HIV-1_{NL4.3} (ref. 12) or 10–20 IU of a single paediatric isolate (pt. Br), a member of the original virus pool. Infection with HIV-1 again resulted in severe depletion of thymocytes, but more slowly than in the first experiment, probably as a result of the lower virus input (Table 1). This depletion was highly significant ($P=0.0009$; Wilcoxon rank sum test). Flow cytometric data suggest that the first thymocytes to be depleted are the CD4⁺/CD8⁺ population, followed by CD4⁺/CD8[−] cells, and subsequently the CD4[−]/CD8⁺ cells.

Quantitative analysis using the polymerase chain reaction (PCR) (Fig. 2a) indicated that the level of viral DNA in infected implants rose rapidly within two to three weeks after infection. One animal (6-11) appeared to achieve, on average, one copy of

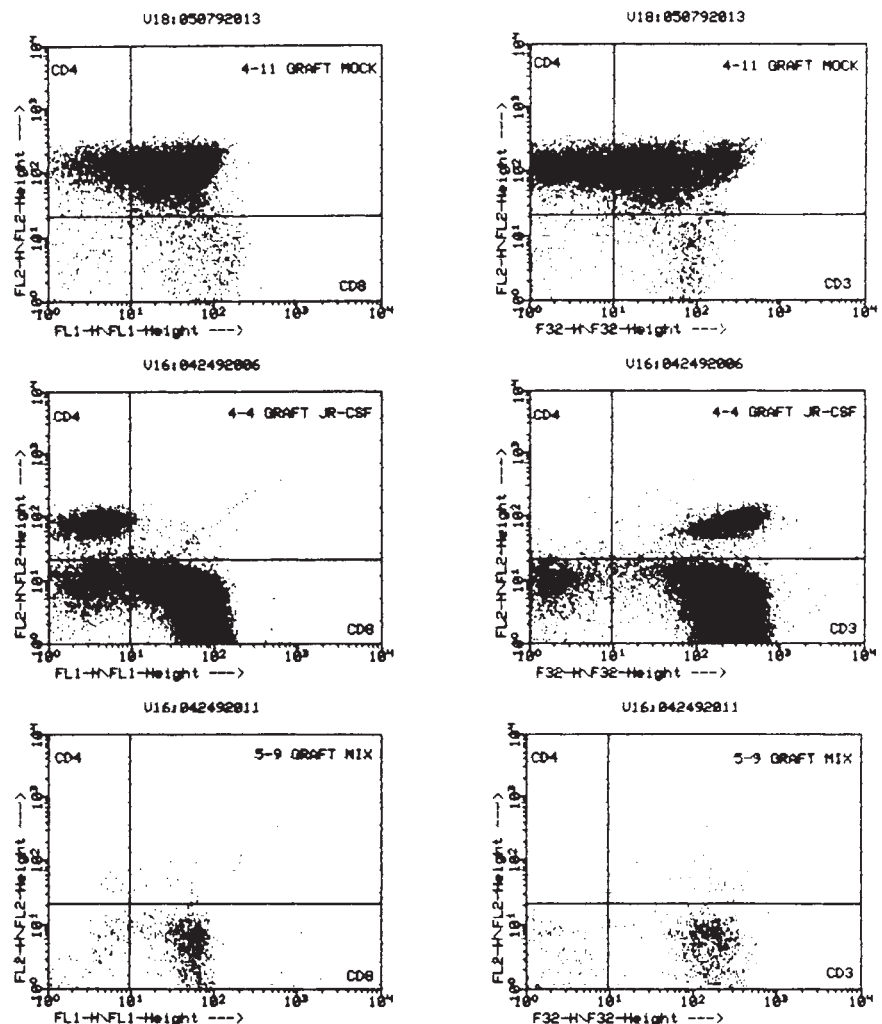
virus for every two thymocytes. The number of proviral genomes subsequently decreased in all animals tested, coincident with the decrease in CD4⁺ cells seen by flow cytometric analysis (Table 1), which is probably due to depletion of infected thymocytes. These data suggest that a high virus load is required for severe thymocyte depletion.

To determine the thymocyte subsets infected, cells from infected implants were sorted, followed by quantitative PCR analysis for viral sequences (Fig. 2b). This analysis was done when CD4⁺/CD8⁺ double-positive cells constituted 60 per cent of total thymocytes. HIV-1 was present almost exclusively in the CD4-bearing populations, with ~5–10-fold more virus being present in the CD4/CD8 double-positive population on a per-cell basis. On a per-organ basis, this population would harbour >90 per cent of the virus, which correlates well with its preferential depletion (Fig. 1). The viral DNA detected in the CD4[−]/CD8⁺ fraction (mouse 8-6) may have arisen in a CD4/CD8 double-positive cell that subsequently matured to the CD4[−]/CD8⁺ phenotype, or alternatively, underwent CD4 downregulation.

Histological analysis also showed striking differences between normal and infected implants. Normal human fetal thymus (Fig. 3a) and control Thy/Liv implants (Fig. 3b) consist of the cortex, which contains immature (CD4/CD8 double-positive) cells, and the medulla, which contains the more mature (CD4 or CD8 single-positive) thymocytes. In contrast, infected implants show no clear demarcation between cortex and medulla owing to cortical depletion of thymocytes. The entire organ appeared to

FIG. 1. Three-colour flow cytometric analysis of Thy/Liv implants from experiment 1. Procedures were performed 5–6 months post-transplantation. Infected implants were analysed by three-colour flow cytometry for human CD3, CD4 and CD8 markers at 17 d and 24 d (control) post-infection. Top panels, data are from an implant receiving heat-inactivated HIV-1 (mouse 4-11); middle panels, from an implant infected with HIV-1_{JR-CSF} (mouse 4-4); and bottom, from an implant infected with pooled paediatric isolates (mouse 5-9). Analysis is shown for CD4 and CD8 (left) and for CD4 and CD3 (right).

METHODS. Thy/Liv cells were stained with monoclonal antibodies (mAbs) (Becton-Dickinson) to human CD8 and CD4 directly conjugated with FITC or phycoerythrin, respectively. The antibody specific for CD3 was conjugated to biotin and secondary-stained with avidin-allophycocyanin. Samples were run on a FAC-Star^{plus} flow cytometer and data analysed using the Lysis II program (Becton-Dickinson). SCID mouse blood was analysed in parallel as a negative control, and isotype control mAbs were also included. Normal human peripheral blood was stained as a positive control in all procedures. Forward versus side scatter plots of control SCID-hu Thy/Liv cells were used to set gates on the live thymocyte population. Five thousand events were acquired, except from infected implants in which there was a severe loss of cellularity. For infections, human Thy/Liv implants were directly infected with ~50 µl of control or virus-containing supernatants⁹. HIV-1_{JR-CSF} and HIV-1_{NL4.3} were recovered following electroporation of COS cells with cloned viral DNA¹⁷. Primary paediatric isolates were recovered following co-cultivation of patient peripheral blood mononuclear cells (PBMC) with normal PHA-stimulated PBMC. Virus was quantitated on normal stimulated PBMC by limiting dilution.



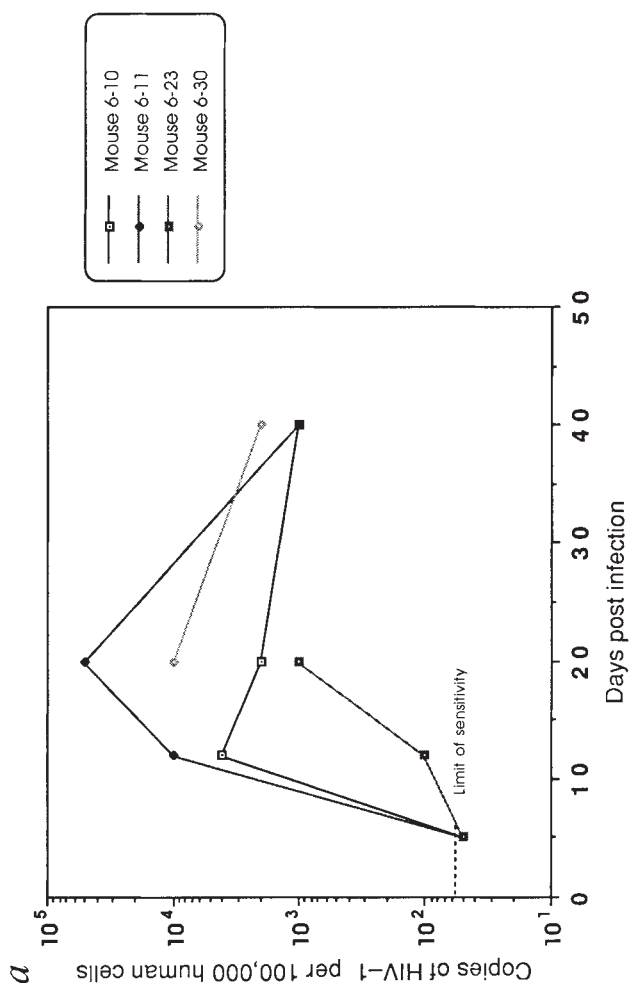


FIG. 2 a, Kinetics of HIV-1 replication in Thy/Liv implants. The number of HIV-1 genomes in 100,000 human thymocytes was determined in implants at various times post-infection. The dotted line at the lower left corner indicates the level of sensitivity of the day 5 PCR analysis, which is due to a limited recovery of cells at this time point.



sequences. Thy/Liv cells were sorted based on surface markers and analysed for virus genomic sequences 28 days post-infection. DNA from 500 cells was analysed in the CD4⁺ and CD4/CD8 double-positive lanes. DNA from 200 cells was amplified in the CD8⁺ lanes. On the right are shown signals from quantitative HIV-1 DNA standards assayed in parallel. Uninfected cells were sorted and analysed in parallel as a negative control. METHODS. a, Sequential biopsies of infected animals were taken on the days indicated. DNA was extracted and analysed by quantitative PCR for HIV-1 genomic sequences using the M667/AA55 primer pair, which detects the R/U5 region of the viral long terminal repeat. HIV-1-specific signal was compared with that of amplified human β -globin sequences to determine the number of virus genomes per human cell equivalent^{18,19}. b, Implants were infected with 200 infectious units of HIV-1_{IR,CSF} 5 months postimplantation. Thymocytes were collected, stained with monoclonal antibodies against CD4 and CD8, and the live cells subjected to cell sorting on a FACStar^{plus}. DNA was subjected to 25 cycles of quantitative PCR amplification using primers specific for the R/U5 region of the viral long terminal repeat^{18,19}. Primers specific for human β -globin were also used to ensure adequate input of DNA (not shown).

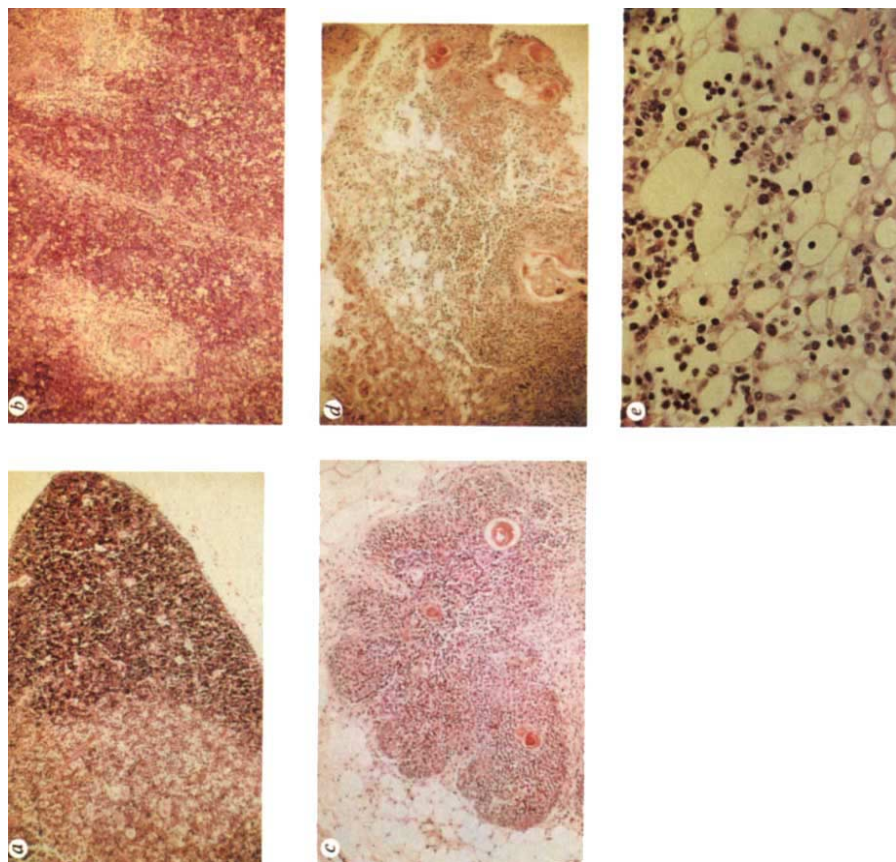


FIG. 3 Histological analysis of treated implants. a, Normal fetal thymus (number 3) (magnification, 97 $\times$); b, control implant (mouse 6-7) 20 d postinoculation (magnification, 97 $\times$); c, implant from animal 4-4 infected with HIV-1_{IR,CSF} 17 d postinfection (magnification, 97 $\times$). The large Hassall's corpuscles are found in many sections of these conjunct organs, and do not appear to be due to HIV-1 infection. d, Implant infected with the paediatric virus pool (number 4-10) 24 d postinfection (magnification, 97 $\times$). e, Implant from animal 4-10 24 d postinfection (387 $\times$) showing pyknotic nuclei. All tissue biopsies were fixed in 10 per cent formalin acetate. 4- μ m paraffin sections were stained with haematoxylin and eosin.

TABLE 1 Summary of the flow cytometric analysis on Thy/Liv implants

Experiment 1				% CD4 ⁺	% CD4 ⁺ /CD8 ⁺	% CD4 ⁺ /CD8 ⁺	% CD8 ⁺
	Mouse ID	Days postinfection	(Quad 1)	(Quad 2)	(Quad 3)	(Quad 4)	
Normal	3-13	N/A	6.0	85.8	1.5	6.7	
	4-17	N/A	11.9	78.2	5.6	4.3	
Heat-inactivated	4-11	24	10.7	84.9	0.9	3.5	
JR-CSF	4-16	15	11.1	79.9	1.4	7.6	
	4-4	17	7.4	0.5	7.5	84.6	
	5-12	17	33.1	42.3	7.2	17.4	
	5-25	24	9.6	75.3	1.0	14.1	
HIV	4-8	17	0.8	0.8	78.6	19.8	
Pool	5-9	17	1.0	2.6	14.1	82.3	
	5-10	17	0.0	0.8	93.6	5.6	
	4-10	24	0.5	4.0	73.5	22.0	
	5-23	24	3.4	5.9	82.2	8.5	

Experiment 2		3 weeks postinfection				5-6 weeks postinfection			
	Mouse ID	% CD4 ⁺	% CD4 ⁺ /CD8 ⁺	% CD4 ⁺ /CD8 ⁺	% CD8 ⁺	% CD4 ⁺	% CD4 ⁺ /CD8 ⁺	% CD4 ⁺ /CD8 ⁺	% CD8 ⁺
		(Quad 1)	(Quad 2)	(Quad 3)	(Quad 4)	(Quad 1)	(Quad 2)	(Quad 3)	(Quad 4)
EP	6-12	1.3	42.7	36.4	19.6	—	—	—	—
	10-1	31.7	48.3	7.7	12.3	13.3	71.3	8.1	7.3
	10-10	28.7	65.7	1.7	3.9	15.1	63.9	17.5	3.5
	11-17	24.4	69.8	1.5	4.3	17.4	71.7	2.1	8.8
	14-1	6.4	86.4	2.8	4.4	8.2	72.8	15.3	3.7
	14-4	9.7	80.9	3.9	5.5	28.3	40.5	6.9	24.3
	14-9	7.6	86.8	2.6	3.0	6.7	87.3	2.8	3.2
	16-14	7.5	86.6	2.2	3.7	—	—	—	—
PHA	6-6	36.1	21.6	36.9	5.4	20.2	63.6	2.2	14.0
	6-7	17.9	76.1	1.2	4.8	11.0	79.8	1.9	7.3
JR-CSF	6-23	22.9	25.1	27.9	24.1	0.5	6.4	93.1	0.0
	6-30	18.0	54.4	2.8	24.8	1.3	0.5	93.8	4.4
Pt. Br	6-10	16.3	71.9	1.7	10.1	0.8	0.7	96.2	2.3
	6-11	0.5	84.8	0.4	14.3	0.7	5.3	92.1	1.9
NL4-3	14-6	6.7	85.2	3.6	4.5	21.0	16.0	1.2	61.8
	14-10	12.3	78.8	2.5	6.4	20.3	1.3	16.8	61.6
	16-2	5.8	85.6	3.3	5.5	11.7	16.9	55.9	15.5
	16-9	9.9	76.7	4.2	9.2	0.0	0.5	98.4	1.1

Implants were infected and analysed at the times indicated, as described for Fig. 1. Animals in experiment 1 received 1,000 IU and were analysed at a single time point; animals in experiment 2 received 200 IU of HIV-1_{JR-CSF} or HIV-1_{NL4-3} or 10–20 IU of the pt. Br isolate. Multiple biopsies were taken in experiment 2: data from biopsies at 3 weeks and 5–6 weeks are shown. 'Normal', implants received no treatment; 'heat-inactivated', implant treated with killed virus (60 °C, 1 h); PHA, implants treated with supernatant from phytohaemagglutinin (PHA)-stimulated uninfected PBMC; EP, implants treated with mock-electroporated COS cell supernatant; JR-CSF and NL4-3, implants infected with cloned virus isolates; HIV pool and pt. Br, implants infected with paediatric virus preparations. Animals 6-12 and 16-14 died after the week 3 biopsy. BALB/c C.B.17 SCID mice obtained from K. Dorshkin were bred at UCLA. Six-to-eight-week-old SCID mice were implanted with human fetal liver and thymus under the left kidney capsule, and with human lymph node into the fourth mammary fat pad, as described^{6,15,16}. Animals were each transplanted with tissues (Advanced Bioscience Resources) from a single donor (gestational ages 16–24 w). The animal identification number preceding the hyphen indicates the particular fetal donor. HIV-1 infection of human lymph nodes was not studied. Control implants <6 mm in diameter gave decreases in the CD4/CD8 double-positive population, whereas larger implants did not, probably because of exhaustion of fetal liver-derived progenitor cells. This phenotype was distinguishable from the effect of HIV infection, as the ratio of CD4⁺ to CD8⁺ cells was not altered in exhausted implants. Therefore only animals with implants ranging from 8–12 mm in diameter were used.

be atrophic and hypocellular (Fig. 3c). Regions of some implants appeared to be nearly entirely depleted of cells and contained thymocytes with pyknotic nuclei (Fig. 3d and 3e). These observations correlate well with the depletion of CD4-bearing cells and the thymocyte subset distribution of viral genomic sequences illustrated in Figs 1 and 2. Other less severe but similar cortical aberrations have been reported in thymuses from HIV-1-infected individuals and fetuses²⁻⁵.

Our data suggest that HIV-1 infection of the SCID-hu mouse reproduces key aspects of HIV-1 pathology in man and may be an important small animal model to study HIV-1-induced pathogenesis *in vivo*. Similar data obtained using a cross-sectional rather than longitudinal analysis support this conclusion¹³. We have been unable to demonstrate killing on this scale in CD4⁺ lymphocytes or in paediatric thymocytes following HIV-1_{JR-CSF} infection *in vitro*¹⁴. Thus, the SCID-hu mouse system does not merely reproduce *in vitro* phenomena,

but allows infection of primary cells to be studied in a more appropriate environment. This model may prove to be important for examining how HIV-1 infection interferes with the ontogeny of the human immune system. □

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Phosphorylation and inactivation of the mitotic inhibitor Wee1 by the *nim1/cdr1* kinase

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THE G2–M phase transition in eukaryotes is regulated by the synergistic and opposing activities of a cascade of distinct protein kinases and phosphatases. This cascade converges on Cdc2, a serine/threonine protein kinase required for entry into mitosis (reviewed in ref. 1). In the fission yeast *Schizosaccharomyces pombe*, inactivation of the Cdc2/cyclin B complex is achieved by phosphorylation of tyrosine 15 by Wee1 (refs 2, 3). The action of the Wee1 kinase is opposed by the action of the Cdc25 phosphatase, which dephosphorylates Cdc2 on tyrosine 15, thereby activating the Cdc2/cyclin B complex^{4–9}. Much less is known about the regulatory signals upstream of *cdc25* and *wee1*. Genetics indicate that the mitotic inducer *nim1/cdr1* acts upstream of *wee1*, possibly as a negative regulator of *wee1* (refs 10, 11). To characterize the *nim1/cdr1* protein (Nim1), we have overproduced it in both bacterial and baculoviral expression systems. We report that Nim1 possesses intrinsic serine-kinase, threonine-kinase and tyrosine-kinase activities. Co-expression of the Nim1 and Wee1 kinases in insect cells results in the phosphorylation of Wee1 and therefore a shift in its electrophoretic mobility on SDS–polyacrylamide gels. When Wee1 is phosphorylated, its ability to phosphorylate Cdc2 on tyrosine 15 is inhibited; treatment with phosphatase restores this kinase activity. Furthermore, purified bacterially produced Nim1 kinase directly phosphorylates and inactivates Wee1 *in vitro*. These results show that *nim1/cdr1* functions as a positive regulator of mitosis by directly phosphorylating and inactivating the mitotic inhibitor Wee1.

To analyse the biochemical activities associated with the *nim1/cdr1* gene product (Nim1), we fused it with the glutathione S-transferase protein (GST) in both bacteria (Fig. 1a) and insect cells (Fig. 1b). GST–Nim1 was phosphorylated both *in vivo* and *in vitro*. In bacteria, phosphoamino-acid analysis revealed primarily phosphoserine, although phosphothreonine and phosphotyrosine were also detected (Fig. 1a). Substitution of arginine for lysine at position 41 generated a kinase-deficient mutant of Nim1 (Fig. 1a). In insect cells, phosphoamino-acid analysis revealed phosphoserine and phosphothreonine when GST–Nim1 was labelled *in vitro* and *in vivo*, whereas phosphotyrosine was only detected when kinase assays were performed *in vitro* (Fig. 1b).

To study the interaction between Wee1 and the Nim1 kinase,

we co-produced them in insect cells. As seen in Fig. 2a (lanes 3 and 4), the electrophoretic mobility of GST–Wee1 is retarded when it is co-produced with GST–Nim1. An identical change in mobility is seen in the assay product from a kinase-deficient mutant of GST–Wee1 (Leu 596 substitution) (ref. 2) in (lanes 1 and 2), suggesting that the shift is due to Nim1-mediated phosphorylation.

A change in electrophoretic mobility of the product was also seen when the kinase domain of Wee1 was fused to GST (GST–Wee1KD) and assayed (Fig. 2b). When insect cells producing GST–Wee1KD (in the absence of GST–Nim1) were incubated with ³²P-orthophosphate, no labelling of GST–Wee1KD was detected (Fig. 2b, lanes 1 and 3). However, co-production of GST–Wee1KD with GST–Nim1 resulted in the labelling of GST–Wee1KD (Fig. 2b, lanes 2 and 4). Phosphoamino-acid analysis of GST–Wee1KD revealed phosphoserine and low levels of phosphothreonine (Fig. 2b). These results suggest that the shift in mobility of Wee1 when co-produced with GST–Nim1 is caused, at least in part, by the phosphorylation of one or more serine residues in the kinase domain of Wee1.

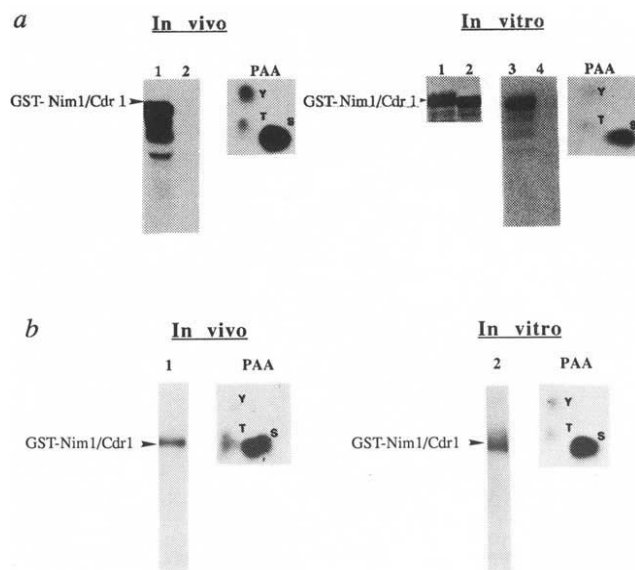


FIG. 1 Expression and phosphorylation of the *nim1/cdr1* protein in bacteria and in insect cells. *In vivo*: Bacteria expressing GST–Nim1 (a, lane 1) or GST (lane 2), and insect cells expressing GST–Nim1 (b, lane 1) were incubated with ³²P-orthophosphate. Proteins were precipitated with glutathione–agarose, resolved by SDS–PAGE on a 7.5% gel and visualized by autoradiography. *In vitro*: Bacteria expressing GST–Nim1 (a, lanes 1 and 3) or a kinase-deficient mutant of GST–Nim1 (lanes 2, 4) and insect cells expressing GST–Nim1 (b, lane 2) were lysed and the proteins precipitated with glutathione–agarose. Kinase assays were done *in vitro*, resolved by SDS–PAGE on a 7.5% gel, and visualized by Coomassie blue staining (a, lanes 1 and 2) or by autoradiography (a, lanes 3 and 4, and b, lane 2). PAA, Two-dimensional phosphoamino-acid analysis of GST–Nim1. S, phosphoserine; T, phosphothreonine, Y, phosphotyrosine.

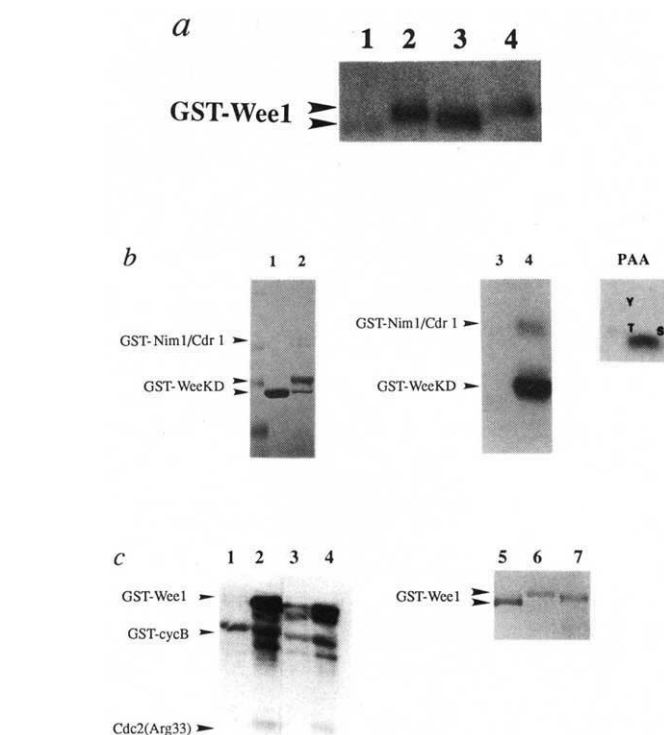
METHODS. pGST–Nim1 was created by digesting pD3 (contains *cdr1* in puc118) with *NdeI*, inserting a *BamHI* linker, and cloning the 2.8-kb *BamHI*/EcoRI fragment into pGEX1 (Pharmacia). pVL–GST–Nim1 was created by changing the EcoNI site of pGST–Nim1 to *XbaI*, excising the GST–Nim1 fusion with *XbaI* and *EcoRI*, and inserting it into pVL1393 (ref. 12). A kinase-deficient form of Nim1 was created by polymerase chain reaction (PCR) amplification of the 170-bp *SphI*–*XhoI* fragment of *nim1/cdr1* using the oligonucleotides: 5'GCCAAGCATGCTAAACTGGTGATTTGGCTGCCATCAGATTATCCC and 5'CGTACTCGAGTGCTAAGTAC. The underlined codon substitutes arginine for lysine 41. The mutated fragment was cloned as an *SphI*/XhoI fragment into GST–nim1. Phosphoamino-acid analysis and all procedures relating to expression and labelling of proteins in bacteria and in insect cells have been described^{2,3,13}.

FIG. 2 Phosphorylation and inactivation of GST-Wee1 by GST-Nim1 in insect cells. **a**, Co-production of GST-Wee1 and GST-Nim1 in insect cells. Insect cells infected with virus encoding GST-Wee1(Leu 596) (lane 1); GST-Wee1(Leu 596) and GST-Nim1 (lane 2); GST-Wee1 alone (lane 3); or GST-Wee1 and GST-Nim1 (lane 4) were labelled *in vivo* with 32 P-orthophosphate. Cells were lysed, proteins were precipitated with glutathione-agarose, resolved by electrophoresis on a 7.5% gel and visualized by autoradiography. **b**, Co-production of GST-Nim1 and the kinase domain of Wee1 in insect cells. Insect cells infected with virus encoding GST-Wee1KD (lanes 1 and 3) or GST-Wee1KD and GST-Nim1 (lanes 2 and 4) were labelled *in vivo*. Cells were lysed and the phosphorylated proteins precipitated with glutathione-agarose, resolved by electrophoresis on a 7.5% gel and visualized by Coomassie blue staining (lanes 1 and 2) and autoradiography (lanes 3 and 4). PAA, Two-dimensional phosphoamino-acid analysis of GST-Wee1KD. Stained proteins left of lane 1 are prestained standards with *M_s* of 218K, 100K, 71K and 43K. **c**, Inhibition of GST-Wee1 by co-production with GST-Nim1. For lanes 1–4, insect cells expressing GST-Wee1 (lane 2), or GST-Wee1 and GST-Nim1 (lanes 3 and 4) were lysed, proteins were precipitated using glutathione agarose and washed with phosphatase buffer. Samples containing GST-Wee1 were incubated in the presence of phosphatase buffer alone; samples containing GST-Wee1 and GST-Nim1 were split in two, one half of which was incubated with phosphatase buffer (lane 3) and the other half with alkaline phosphatase (lane 4). Samples were then washed, purified Cdc2(Arg 33)/cyclin B complexes were added and kinase reactions assayed *in vitro*. Purified Cdc2(Arg 33)/cyclin B complexes were also assayed alone (lane 1). Proteins were resolved by SDS-PAGE on a 12% gel and visualized by autoradiography. Lanes 5–7: GST-Wee1 was produced alone (lane 5) or co-produced with GST-Nim1 (lanes 6, 7) and affinity-purified using glutathione-agarose. GST-Wee1 was incubated in phosphatase buffer alone (lanes 5, 6) or in the presence of alkaline phosphatase (lane 7). Proteins were resolved on a 7% gel and Wee1 visualized by immunoblotting with anti-Wee1 serum.

METHODS. A baculoviral vector encoding GST-Wee1KD was created by changing the second *Nco*I site of pGEX2T-Wee1 (ref. 13) to *Bam*HI using the linker 5'CATGGCGGATCCGC. The *Bam*HI flanked kinase domain of *wee1* was then inserted into the *Bam*HI site of GST-pVL (a baculoviral expression vector containing the GST gene). A baculoviral vector encoding the kinase-deficient mutant of *wee1* fused to GST was created as follows: plasmid pWG-L596 (ref. 14) was digested with *Kpn*I and *Bgl*II, and the 1.2-kb fragment substituting leucine for lysine at 596 was

To determine whether the phosphorylation of GST-Wee1 that occurs when it is co-produced with GST-Nim1 affects the kinase activity of Wee1, we tested its ability to phosphorylate the Cdc2/cyclin B complex *in vitro*. Although the faster-migrating form of GST-Wee1 readily phosphorylated Cdc2 in this assay (Fig. 2c, lane 2), there was no phosphorylation of Cdc2 by the slower-migrating form of GST-Wee1 (produced in the presence of GST-Nim1) (Fig. 2c, lane 3). Treatment of the slower form of GST-Wee1 with alkaline phosphatase not only increased its electrophoretic mobility (Fig. 2c, lane 7), but also restored its

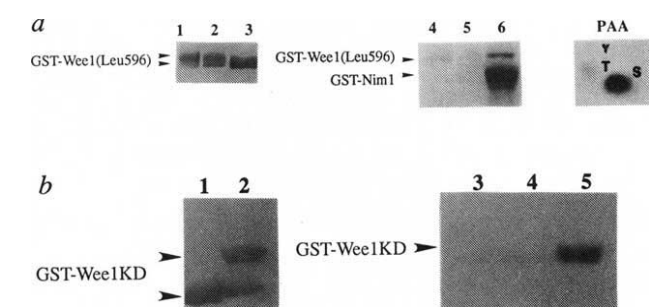
FIG. 3 Phosphorylation of GST-Wee1 *in vitro* by GST-Nim1. **a**, Phosphorylation of a kinase-deficient mutant of Wee1 *in vitro* by Nim1. Lanes 1–3: GST-Wee1(Leu 596) was produced and precipitated from insect cells using glutathione-agarose. Kinase reactions were for 60 min (lane 1) or 30 min (lane 2). Alternatively, Wee1 was untreated (lane 3). Proteins were resolved by SDS-PAGE on a 7% gel and visualized by immunoblotting with anti-Wee1 serum. Lanes 4–6: purified GST-Wee1(Leu 596) was incubated with buffer alone (lane 4), with a kinase-deficient mutant of GST-Nim1 (lane 5), or with the active kinase GST-Nim1 (lane 6), and kinase activity assayed. Proteins were resolved by electrophoresis through a 10% gel and visualized by autoradiography. PAA, Two-dimensional phosphoamino-acid analysis of GST-Wee1(Leu 596) labelled *in vitro*. **b**, Phosphorylation of the kinase domain of Wee1 *in vitro* by Nim1. Lanes 1 and 2: GST-Wee1KD was produced and purified from insect cells using glutathione-agarose. Affinity-purified GST-Wee1KD was then incubated with either a kinase-deficient mutant of GST-Nim1 (lane 1) or kinase-active GST-Nim1 (lane 2). Proteins were resolved by electrophoresis through a 7% gel and visualized by immunoblotting with anti-Wee1 serum. Lanes 3–5: GST-Wee1KD was



excised and cloned into the *Kpn*I/*Bgl*II sites of pGEX2T-Wee1 (ref. 13). The GST-Wee1(Leu 596)-containing plasmid was then digested with *Xba*I and *Nhe*I and the 3.5-kb fragment containing Wee1(Leu 596) fused to GST was inserted into the *Xba*I site of pVL1393 (ref. 12). Methods outlining the purification of Cdc2/cyclin B complexes, their phosphorylation by GST-Wee1 and phosphoamino-acid analysis have been described^{3,13}. Alkaline phosphatase treatment: samples were washed twice with buffer consisting of 50 mM Tris, pH 8.5, 1 mM DTT, then 10 U alkaline phosphatase (Boehringer Mannheim) were added and the reactions were incubated at 30°C for 30 min, washed with kinase buffer, and the kinase reactions assayed as described^{3,13}.

ability to phosphorylate Cdc2 (Fig. 2c, lane 4).

To determine whether Wee1 is a direct substrate of the Nim1 kinase, we developed an *in vitro* assay system (Fig. 3). There was a shift in the electrophoretic mobility of GST-Wee1 (Leu 596) upon incubation with bacterially produced GST-Nim1 *in vitro* (Fig. 3a, lanes 1–3). Also, when GST-Wee1 (Leu 596) was assayed either alone (Fig. 3a, lane 4) or with a kinase-deficient mutant of GST-Nim1 (Fig. 3a, lane 5), little phosphorylation of Wee1 was evident. In contrast, when GST-Wee1 (Leu 596) was incubated with kinase-active GST-Nim1, Wee1 was phos-



produced and purified from insect cells using glutathione-agarose. Kinase reactions were then assayed in the presence of buffer alone (lane 3), a kinase-deficient mutant of GST-Nim1 (lane 4) or kinase-active GST-Nim1 (lane 5). Proteins were resolved by SDS-PAGE on a 9% gel and visualized by autoradiography. Methods were as described^{2,3,13}.

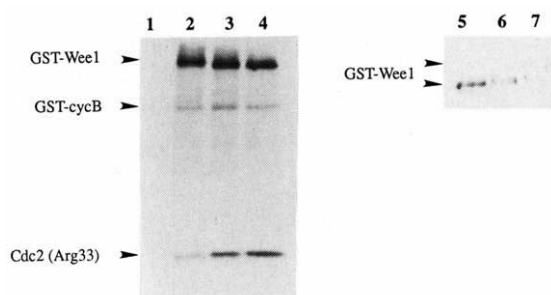


FIG. 4 Inactivation of GST-Wee1 *in vitro* by GST-Nim1. Glutathione-agarose-bound GST-Wee1 was incubated with either kinase-active (lanes 2, 3, 6, 7) or kinase-deficient GST-Nim1 (lanes 4, 5). Reactions were washed into phosphatase buffer either in the absence (lanes 2, 7) or presence of alkaline phosphatase (lanes 3–6). One half of each reaction was assayed for its ability to phosphorylate Cdc2(Arg 33)/cyclin B complexes *in vitro* (lanes 2–4); the other half was monitored for the electrophoretic mobility of Wee1 (lanes 5–7). Purified Cdc2(Arg 33)/cyclin B complexes were also assayed alone (lane 1). Proteins were resolved by SDS-PAGE on a 10% gel and visualized by autoradiography (lanes 1–4) or on a 7.5% gel and visualized by immunoblotting with anti-Wee1 serum (lanes 5–7).

METHODS. To assay for the ability of insect cell-derived GST-Wee1 to phosphorylate Cdc2/cyclin B complexes, glutathione-agarose-bound GST-Wee1 was incubated with bacterially produced GST-Nim1 or a kinase-deficient mutant of GST-Nim1 and 1 mM ATP for 40 min at 30 °C. Samples were washed twice with buffer consisting of 50 mM Tris, pH 8.5, 1 mM DTT, and in some cases 10 U alkaline phosphatase were added and reactions incubated at 30 °C or 30 min. Reactions were washed and then incubated with either purified Cdc2(Arg 33)/GST-cyclin B complexes or with a lysate containing Cdc2(Arg 33)/GST-cyclin B complexes. Complexes were washed and kinase assayed^{2,3}.

phorylated (Fig. 3a, lane 6). Phosphoamino-acid analysis revealed phosphoserine and low levels of phosphothreonine. Similar results were obtained when the kinase domain of Wee1 (GST-Wee1KD) was assayed. Incubation of GST-Wee1KD with bacterially produced GST-Nim1 *in vitro* resulted in the phosphorylation of GST-Wee1KD (Fig. 3b, lanes 3–5) and a shift in its electrophoretic mobility (Fig. 3b, lanes 1 and 2).

This purified *in vitro* system was also used to test the functional effects of the Nim1-mediated phosphorylation of Wee1. As seen in Fig 4, there is a stoichiometric shift in the electrophoretic mobility of GST-Wee1 upon phosphorylation by kinase-active GST-Nim1 *in vitro* (lanes 5 and 7). When Wee1 is itself phosphorylated, it loses its ability to phosphorylate its physiological substrate Cdc2 (lanes 2 and 4); treatment with phosphatase restores both the electrophoretic mobility and the kinase activity of Wee1 (lanes 3 and 6).

Our results provide direct evidence that Wee1 is phosphorylated and inactivated by the Nim1 kinase. We have shown previously that phosphorylation of Cdc2 on tyrosine 15 by Wee1 completely inhibits the activity of the Cdc2/cyclin B complex^{2,3}, and now we have demonstrated that Nim1 kinase phosphorylates Wee1 and thereby ablates the activity of Wee1 towards Cdc2. Thus the Nim1 kinase functions as a mitotic inducer by negatively regulating the mitotic inhibitor Wee1. □

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Nim1 kinase promotes mitosis by inactivating Wee1 tyrosine kinase

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IN most species, including the fission yeast *Schizosaccharomyces pombe*, the Cdc2/cyclin B mitosis-inducing kinase is maintained in an inhibited state during interphase as a result of phosphorylation of a tyrosine residue in the ATP-binding region of Cdc2 (refs 1–3). This site is phosphorylated by Wee1 kinase^{4–9} and dephosphorylated by Cdc25 phosphatase^{10–15}. In fission yeast an additional element of the G2/M control Nim1/Cdr1 kinase, has been identified which functions as a potent mitotic inducer^{16,17}. These studies suggested that Nim1 acts by inhibiting Wee1, perhaps by direct phosphorylation. Consistent with this model, we report here that Wee1 is hyperphosphorylated in cells that overproduce Nim1. Likewise, Wee1 phosphorylation is reduced in *nim1*[–] cells. Highly purified Nim1 kinase phosphorylates Wee1 *in vitro*, resulting in strong inhibition of Wee1 kinase. These observations show that Nim1 promotes the onset of mitosis by inhibiting Wee1.

Overexpression of *nim1*⁺ advances mitosis, causing the wee phenotype in which cells divide when they are half the size of wild type¹⁶. Wee phenotypes caused by overexpression of *nim1*⁺ and inactivation of *wee1*⁺ are not additive, suggesting that Nim1 acts as an inhibitor of Wee1. According to our model, overproduction of Nim1 and inactivation of Wee1 should exhibit similar phenotypes when combined with other genetic alterations affecting mitotic control. We constructed strains expressing either *nim1*⁺, or an active form of Nim1 that lacks the C-terminal 239 amino acids (*nim1*-Cat) or a form of Nim1 predicted to be catalytically inactive (*nim1*-K41A)¹⁸. *Nim1*⁺ and *nim1*-Cat overexpression caused a wee phenotype, whereas *nim1*-K41A overexpression had no effect, indicating that the protein kinase activity of Nim1 is essential for its function as a mitotic inducer. Having done these control experiments we tested the first of two crucial predictions that follow from the model, namely that the wee phenotypes caused by overexpression of *nim1*⁺ and *cdc25*⁺ should be additive. This prediction is based on the finding that loss of Wee1 activity in a *cdc25*⁺ overproducer background results in mitotic catastrophe, namely the lethal premature induction of mitosis¹⁰. Induction of *nim1*⁺ or *nim1*-Cat overexpression in a strain that also overproduced Cdc25 was lethal, causing mitotic catastrophe, whereas *nim1*-K41A overexpression caused no phenotype (Fig. 1b). In the second test we examined the interaction between *nim1*⁺ overexpression and inactivation of *mik1*⁺. Mik1 and Wee1 kinases share an essential function⁶. The model predicts that *nim1*⁺ overexpression should be lethal in a strain lacking Mik1. Indeed, we found that induction of *nim1*⁺ and *nim1*-Cat caused mitotic catastrophe in a *mik1*[–] *ura4* background, whereas *nim1*-K41A overexpression had no effect

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(Fig. 1b). These tests indicate that Nim1 acts as a mitotic inducer by inhibiting Wee1.

To determine whether Nim1 can directly phosphorylate and inactivate Wee1, we investigated the phosphorylation state of Wee1 in a strain lacking Nim1, a *nim1*⁺ strain, and a Nim1 overproducer. Phosphorylation of Wee1 in cells lacking Nim1 was reduced by ~50 per cent compared to Wee1 from *nim1*⁺ cells (Fig. 2a). This decreased Wee1 phosphorylation was associated with a faster mobility in SDS-PAGE. In the Nim1-overproducer strain, the level of Wee1 phosphorylation was increased ~2-fold compared to *nim1*⁺ cells (Fig. 2a). Likewise, the increased Wee1 phosphorylation seen in the Nim1 overproducer correlated with a reduced mobility of the protein in SDS-PAGE. Wee1 protein from Nim1-overproducer cells was phosphorylated equally on serine and threonine; no phosphotyrosine was detected. Nim1 was present as a ~70–80K phosphoprotein which was phosphorylated predominantly on serine and to a lesser extent on threonine (Fig. 2).

These results support our model by showing that Wee1 phos-

phorylation is significantly affected by Nim1 *in vivo*. Nim1 and Wee1 were next made in Sf9 cells, an insect expression system¹⁹. These proteins were fused to a leader peptide containing consecutive histidine residues, allowing purification by Ni-NTA affinity chromatography²⁰. Both proteins became labelled with ³²P in autophosphorylation assays (Fig. 3a). Wee1 was phosphorylated equally on serine and tyrosine, consistent with earlier observations⁵. Nim1 was phosphorylated predominantly on serine and threonine, although a low level of phosphotyrosine was also consistently found. The tyrosyl kinase activity of Nim1 is unexplained, as neither it nor Wee1 are phosphorylated on tyrosine at detectable levels *in vivo*. Nim1 has restricted substrate specificity, as it failed to phosphorylate a battery of commonly used substrates, including histone H1, enolase, casein and angiotensin II.

If Nim1 directly regulates Wee1, then Nim1 should promote Wee1 phosphorylation when both proteins are co-produced in

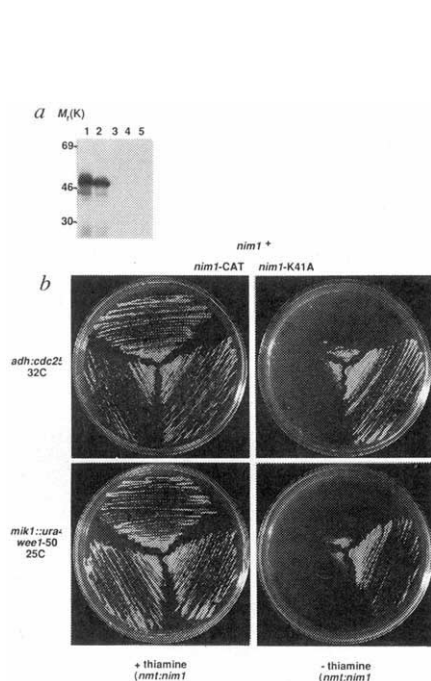


FIG. 1 Nim1 overproduction is lethal in cells that overproduce Cdc25 or lack Mik1. *a*, Immunoblot analysis using anti-Nim1 antibody of extracts of a *leu1-32* strain transformed with plasmids having *nim1*-Cat or *nim1*-K41A under the control of the thiamine-repressible *nmt1* promoter²³. Lane 1, *nim1*-Cat transformants grown without thiamine; lane 2, *nim1*-K41A transformants grown without thiamine; lane 3, *nim1::LEU2* control; lane 4, *nim1*-Cat transformants grown with thiamine; lane 5, *nim1*-K41A transformants grown with thiamine. Nim1-Cat and Nim1-K41A are detected as ~49K species (lanes 1 and 2). *b*, Growth of transformants under the conditions indicated. Each Petri dish contained cells transformed with plasmids having (clockwise from top): *nmt1::nim1*⁺, *nmt1::nim1*-K41A and *nmt1::nim1*-Cat. Induction of *nim1*⁺ or *nim1*-Cat was lethal in cells that overproduce Cdc25 (*adh::cdc25*⁺) or lack Mik1 (*mik1::ura4*⁺), whereas *nim1*-K41A had no effect. Note that *mik1::ura4*⁺ *wee1*-50 transformants were incubated at 25 °C, a permissive temperature for *wee1*-50.

METHODS. *a*, *Nim1*-K41A was made by sequential polymerase chain reaction (PCR) mediated mutagenesis, changing codon 41 from AAA to GCG. After PCR, DNA sequencing confirmed that no additional mutations had been introduced. Full-length *nim1*⁺ and *nim1*-Cat constructs were generated by PCR and cloned at *Bgl*II–*Not*I fragments into a modified pREP1 plasmid (pREP1X6HHA; P.R., unpublished result). Methods of molecular and biochemical analysis of fission yeast have been described²⁴. Antibodies were raised against purified Nim1-Cat protein produced in bacteria. *b*, Transformant cells were patched from media containing thiamine onto the centre region of plates as shown, incubated for 16 h and then streaked out.

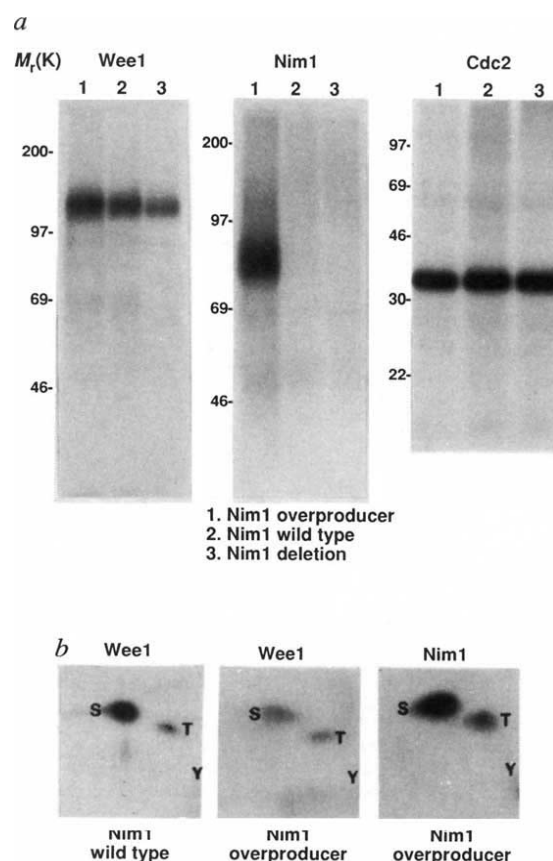


FIG. 2 Wee1 phosphorylation level *in vivo* is regulated by Nim1, Nim1-overproducer (*adh::nim1*⁺), *nim1*⁺, and Nim1-deletion (*nim1::LEU2*) strains, harbouring *adh::wee1*-50, were metabolically labelled with ³²PO₄. *a*, SDS-PAGE analysis of Wee1, Nim1 and Cdc2. Both the phosphorylation level and the mobility of Wee1 is strongly influenced by Nim1 activity. Nim1 was detected as a ~70–80K phosphoprotein in the Nim1-overproducer cells. Long exposure of the autoradiogram revealed an identically sized phosphoprotein in the extract from *nim1*⁺ cells which was absent in *nim1::LEU2* cells (not shown). The Cdc2 immunoprecipitates ensured that roughly equal amounts of a control phosphoprotein were recovered from the three strains. Immunoblots confirmed that Wee1 was present at equal levels in the three strains (data not shown). *b*, Two-dimensional phospho-amino-acid analysis of Nim1 and Wee1 proteins.

METHODS. Strain VG2 (ref. 4), which has an integrated copy of *adh::wee1*-50, was crossed with *adh::nim1*⁺ and *nim1::LEU2* strains¹⁶. *In vivo* labelling with ³²P inorganic phosphate, immunoprecipitation and 2-D phospho-amino-acid analysis was performed as described⁵. Labelling was done for 4 h at 25 °C following a shift from 35.5 °C.

Sf9 cells. As detected by immunoblotting, co-production with Nim1 caused a quantitative shift of Wee1 to a slower species (Fig. 3b). An *in vivo* labelling experiment using $^{32}\text{PO}_4$ demonstrated that the reduction of Wee1 mobility caused by Nim1 was accompanied by a ~five-fold increase in Wee1 phosphorylation (data not shown). To test whether functional properties of Wee1 were altered by Nim1-induced phosphorylation, we measured Wee1 kinase activity using the peptide angiotensin II, a preferred artificial substrate of both fission yeast and human Wee1 (refs 5, 7). As shown in Fig 3c, the kinase activity of Wee1 purified from cells expressing both Wee1 and Nim1 was decreased ~10-

fold relative to Wee1 from singly infected cells.

We next determined whether Nim1 is directly responsible for phosphorylation and inactivation of Wee1. Nim1 and Wee1 were separately purified from Sf9 cells and then incubated together in kinase buffer. Nim1 caused a marked reduction in the mobility of Wee1 in SDS-PAGE (Fig. 4a, lanes 1 and 2). This effect was dependent on the addition of ATP, was not changed by prior treatment of Wee1 with the irreversible kinase inhibitor 5'-*p*-fluorosulphonylbenzoyl-adenosine (FSBA),²¹ but was abolished by treatment of Nim1 with FSBA (data not shown). These results do indicate that Nim1 is directly responsible for Wee1

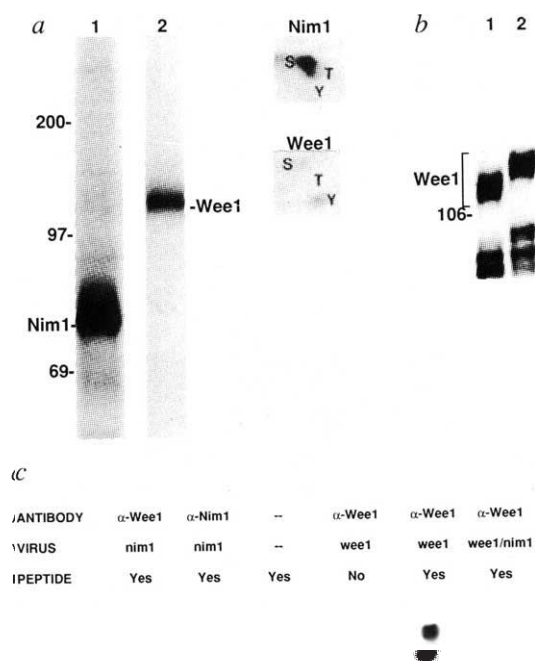


FIG. 3 Nim1 promotes phosphorylation and inactivation of Wee1 in Sf9 cells. *a*, *In vitro* autophosphorylation of immunoprecipitated Wee1 and Nim1 produced in Sf9. No phosphoprotein was detected in control samples in which Wee1 lysates had been treated with anti-Nim1 antibodies or Nim1 lysates that had been treated with anti-Wee1 antibodies (data not shown). *b*, Immunoblot analysis of Wee1 from cells expressing Wee1 alone (lane 1) or co-expressing Wee1 and Nim1 (lane 2). Note that co-expression with Nim1 caused a quantitative shift of Wee1 to a slower-migrating species. *c*, Wee1 was immunoprecipitated from Sf9 lysates and tested in kinase assays using the peptide substrate angiotensin II, which is phosphorylated on tyrosine by Wee1 (refs 5, 7). Products of kinase assays were separated by electrophoresis on a thin-layer chromatography plate and imaged by autoradiography. Controls demonstrate that angiotensin II phosphorylation is dependent on addition of active Wee1 to the assay. The activity of Wee1 protein isolated from Wee1/Nim1 co-infected cells was decreased to ~10% of that measured for protein isolated from Wee1-infected cells. **METHODS.** Wee1¹ and nim1¹ were cloned into the *Bam*HI site of pBlueBacHisA (Invitrogen, San Diego). The Wee1 construct lacked the N-terminal 10 amino acids⁴. The Nim1 construct was full-length¹⁷. Recombinant viruses were isolated according to the manufacturer's methods. The multiplicity of infection was ~10 and cells were collected 60 h after infection. Immune complex kinase assays have been described⁵; kinase assays using angiotensin II (Calbiochem), as a substrate were done as described^{5,7}. Samples (5 μ l) were loaded on a cellulose plate and electrophoresed at 1,000 V for 75 min in buffer at pH 1.9 (ref. 25).

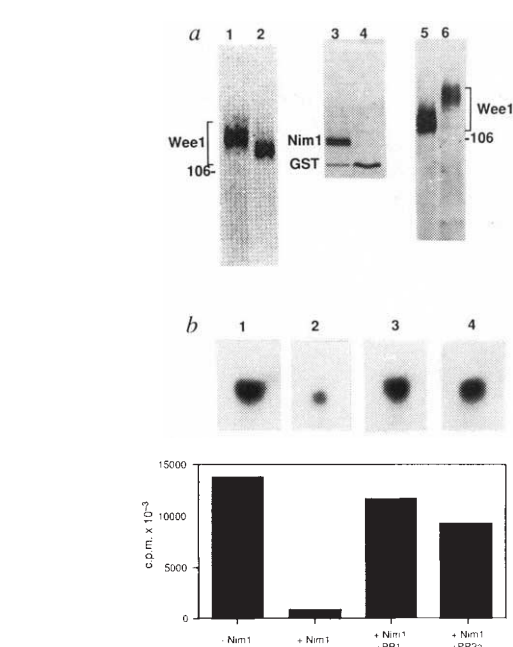


FIG. 4 Wee1 is phosphorylated and inactivated by Nim1 *in vitro*. *a*, Immunoblot of Wee1 purified from Sf9 lysates by Ni-NTA chromatography which was incubated either with Ni-NTA-purified Nim1 (lane 1) or alone (lane 2). In a separate experiment, Wee1 was incubated with GST-Nim1 purified from bacteria. Lane 3 shows Coomassie blue-stained SDS-PAGE gel of Nim1 that had been cleaved from the GST moiety; lane 4 shows GST alone. Ni-NTA-purified Wee1 was incubated either alone (lane 5) or with GST-Nim1 (lane 6) and analysed by immunoblotting. *b*, Angiotensin II phosphorylation assay of untreated Wee1 (lane 1), Wee1 phosphorylated by GST-Nim1 (lane 2), Wee1 phosphorylated by GST-Nim1 and then dephosphorylated with either PP1 or PP2a (lanes 3 and 4 respectively). **METHODS.** Ni-NTA chromatography was done according to the manufacturer's protocol (Qiagen, Chatsworth). For *in vitro* phosphorylation, Wee1 and Nim1 bound to Ni-NTA were equilibrated separately or together in kinase buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM DTT). The reaction mix was adjusted to 100 mM imidazole, 2 mM ATP and the assay was terminated after 30 min at 30 °C. A DNA fragment encoding Nim1-Cat was cloned into a modified pGEX vector. Soluble GST-Nim1 was purified by glutathione-Sepharose chromatography according to the manufacturer's methods (Pharmacia). GST-Nim1 was cleaved using thrombin as recommended (Pharmacia). Wee1 bound to Ni-NTA was incubated either alone or with GST-Nim1 bound to glutathione in kinase buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 10 mM MnCl₂, 20 mM glutathione, 1 mM DTT, 2 mM ATP, 1 μ M okadaic acid) for 40 min at 30 °C. Samples were washed ten times with washing buffer (50 mM Tris, pH 7.5, 0.2% NP40, 50 mM NaF, 200 mM NaCl, 1 mM sodium vanadate, 1 mM DTT), twice with kinase buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 10 mM MnCl₂, 20 mM NaF, 1 mM sodium vanadate, 1 mM DTT) and then assayed as described. Kinase assays were quantified using an AMBIS system. PP1 and PP2a were purified from rabbit skeletal muscle²⁶.

phosphorylation, but do not eliminate the possibility that Nim1 produced in Sf9 cells co-purifies with another kinase that phosphorylates Wee1. We therefore produced active Nim1-Cat in bacteria as a glutathione *S*-transferase (GST) fusion protein. GST-Nim1, purified to near homogeneity, caused the mobility shift of Wee1 in SDS-PAGE (Fig. 4a, lanes 3–6). The kinase activity of Wee1 that had been phosphorylated by Nim1 was reduced ~15-fold (Fig. 4b); Wee1 activity was restored by treatment with protein serine/threonine phosphatases. Protein phosphatase 1 (PP1) treatment increased Wee1 kinase activity ~13-fold; protein phosphatase 2A (PP2A) treatment increased activity ~10-fold (Fig. 4b).

These results establish that Nim1 can inhibit Wee1 by direct phosphorylation. These *in vitro* studies, coupled with *in vivo* experiments showing that Wee1 phosphorylation correlates with Nim1 activity, and that Nim1 overproduction is functionally equivalent to mutational loss of Wee1 function, provide conclusive evidence that Nim1 acts as an inducer of mitosis by directly phosphorylating Wee1 and inhibiting its tyrosine kinase activity. The fact that Nim1 inhibits the ability of Wee1 to phosphorylate an artificial substrate unrelated to Cdc2 indicates that the mechanism of inhibition involves direct inactivation of Wee1 kinase function, rather than reduction of affinity for Cdc2 for example. The sites of Wee1 that are phosphorylated by Nim1 are as yet unknown, but ~55K N-terminal truncated forms of Wee1 remain sensitive to Nim1 inhibitor *in vivo* (unpublished data), consistent with a mechanism by which Nim1 phosphorylates the catalytic domain of Wee1. A functional homologue of Wee1 in human cells has been reported^{7,22}, but no homologue of Nim1

has been found in any species. But, given the remarkable conservation of mitotic control between yeast and man, Nim1 would be expected to be a generally conserved mitotic regulator □

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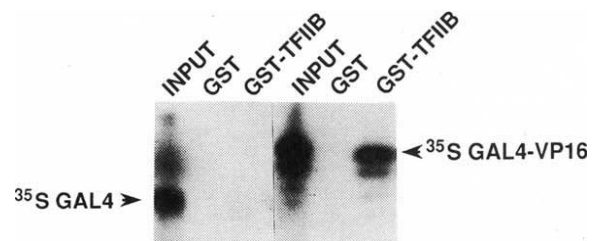
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Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation

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How eukaryotic promoter-specific activator proteins (activators) stimulate transcription is a central question. We have previously shown that an acidic activator can directly interact with the general transcription factor TFIIB and increase its stable assembly into a preinitiation complex^{1,2}. We have proposed that this increase in TFIIB assembly is at least part of the mechanism by which an acidic activator functions^{1,2}. A prediction of this hypothesis is that a TFIIB mutant unable to interact with an acidic activator could not support activated transcription, and here we present experiments that verify this prediction. In conjunction with previous studies, our results argue that interaction between an acidic activator and TFIIB is necessary for transcriptional activation.

We have shown previously that TFIIB can bind directly and selectively to an immobilized VP16 acidic activation region^{1,2}. In this study we sought to design discrete TFIIB mutants defective for interaction with acidic activation domains. This required delineation of the TFIIB region that is necessary for this interaction. To facilitate the analysis of multiple TFIIB derivatives, we developed an assay in which TFIIB, rather than

FIG. 1 Specific interaction between immobilized TFIIB and the transcriptional activation domain of VP16. ³⁵S-methionine-labelled GAL4(1–94)–VP16 and as a control, GAL4(1–94) (lanes marked INPUT) were incubated with either glutathione-S-transferase (GST) or GST–TFIIB. After extensive washing the bound proteins were eluted, resolved by SDS–PAGE and visualized by autoradiography.

METHODS. The coding sequence of human TFIIB was cloned into pGEX 2T vector and GST fusion proteins prepared as described previously¹. One microgram of GST–TFIIB coupled to 20 μ l glutathione agarose beads was incubated at 4°C in 0.6 ml of buffer containing 150mM KCl, 40mM HEPES, pH 7.5, 0.5mM EDTA, 5mM MgCl₂, 1mM DTT, 1mM PMSF, 0.5 mg ml^{–1} bacterial protein extract and 0.05% NP40 for 1 hour. Ethidium bromide (400 μ g ml^{–1}) was included to prevent interactions involving DNA³. Following this incubation, ³⁵S-methionine labelled GAL4(1–94) or GAL4(1–94)–VP16 was added and binding allowed to proceed for 1 h. The beads were then washed five times with the same buffer and bound proteins eluted with SDS–PAGE loading buffer. The proteins were resolved by SDS–PAGE (10%) and visualized by autoradiography. GAL4(1–94) and GAL4(1–94)–VP16 were cloned into pGEM-3 and mRNA transcribed with SP6 RNA polymerase. The mRNA was translated in wheat germ extract incorporation ³⁵S-labelled methionine as described by the manufacturer (Promega).

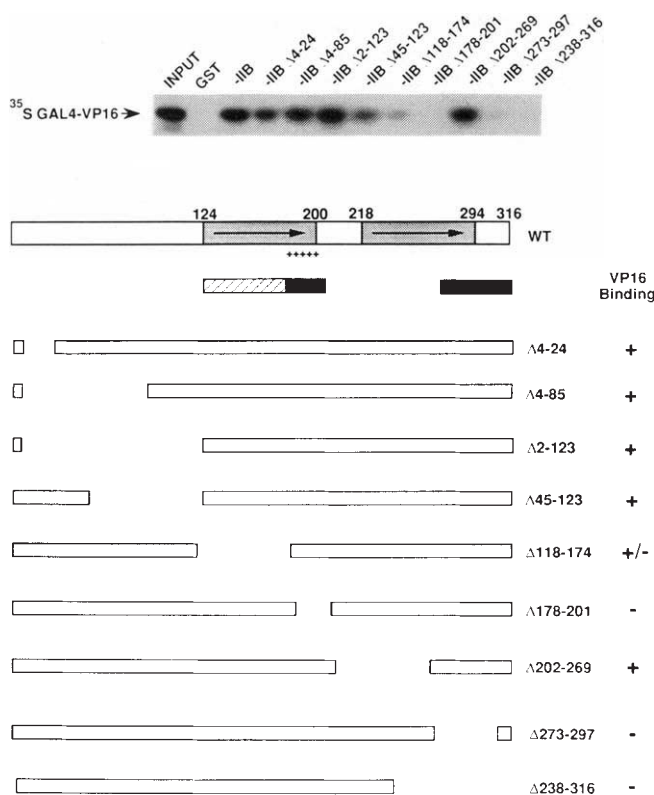


FIG. 2 Two separable domains within TFIIB are required for interaction with the VP16 acidic activation domain. Deletion mutants of TFIIB were expressed as GST fusion proteins and coupled to glutathione agarose beads so that the final concentration was 1 μg of fusion protein per 20 μl glutathione agarose beads. Aliquots of the resins were analysed by SDS-PAGE to confirm the concentration and integrity of the bound proteins (data not shown). Binding to GAL4(1-94)-VP16 was as described in Fig. 1, except that ethidium bromide was omitted. A schematic diagram of wild-type TFIIB and the TFIIB deletion mutants are shown below the autoradiogram. The regions of TFIIB required (black box) or that augment (striped box) binding to VP16 is indicated.

VP16, was the immobilized ligand. A glutathione-S-transferase (GST) fusion of human TFIIB (GST-TFIIB) was constructed and the fusion protein immobilized on glutathione-agarose beads. The GST-TFIIB beads were incubated either with *in vitro* translated ^{35}S -labelled GAL4 (1-94)-VP16 (GAL4-VP16) or, as a control, ^{25}S -labelled GAL4 (1-94) (GAL4). After extensive washing, the bound proteins were eluted, fractionated on an SDS-polyacrylamide gel, and visualized by fluorography. The results in Fig. 1 show that neither GAL4 derivative bound to the control GST beads, whereas GAL4-VP16 but not GAL4 bound to GST-TFIIB. Thus, as expected^{1,2} this protein-protein interaction is between TFIIB and the VP16 acidic activation region. Inclusion of ethidium bromide did not affect binding, indicating that the VP16-TFIIB interaction was not due to contaminating DNA³. These data confirm our previous studies^{1,2} in which VP16, rather than TFIIB, was the immobilized ligand.

To delineate the region of TFIIB required for interaction with VP16, we constructed a series of in-frame TFIIB deletion mutants. Each TFIIB derivative was fused to GST, and the resulting GST-TFIIB fusion proteins analysed for binding to GAL4-VP16. Human TFIIB has an N-terminal tail, and two imperfect direct repeats, the first of which contains at its C terminus a potential positively charged amphipathic α -helix^{4,5}. The results of Fig. 2 show that deletion of the N-terminal 123 amino acids of TFIIB did not significantly affect binding of GAL4-VP16 ($\Delta 4-24$, $\Delta 4-85$, $\Delta 2-123$, $\Delta 45-123$). An in-frame deletion within the N-terminal portion of the first repeat compromised GAL4-VP16 binding ($\Delta 118-174$), and deletion of the putative amphipathic α -helix within the first repeat eliminated binding ($\Delta 178-201$). Deletions within the C-terminal portion of the second repeat also abolished binding ($\Delta 273-297$ and $\Delta 238-316$). We conclude that two distinct regions of TFIIB, contained within residues 178-201 and 269-316, are required for the interaction with the VP16 acidic activation region.

Each of these TFIIB in-frame deletion mutants was tested in an *in vitro* transcription assay and found to be completely defective in supporting an activator-independent 'basal' trans-

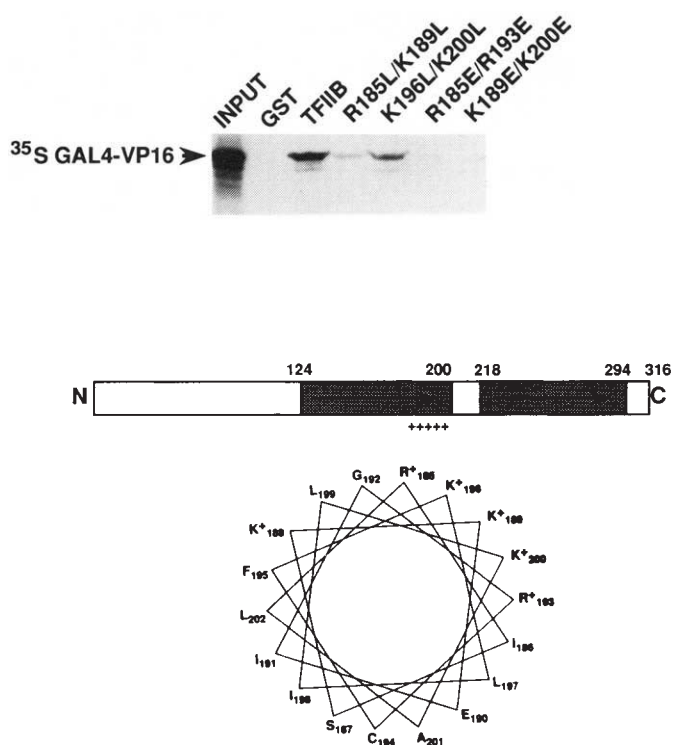


FIG. 3 Double amino-acid substitution mutations in the basic region of TFIIB decrease binding to the VP16 acidic activation region. Amino-acid substitution mutants of TFIIB were expressed as GST fusion proteins and assayed for binding to GAL4(1-94)-VP16 as described in Fig. 1. In this experiment, TFIIB or its derivatives were fused to GST at amino acid position 124. A schematic diagram of TFIIB and a helical wheel depiction of the putative amphipathic basic α -helix are also shown.

cription reaction (data not shown). The most likely interpretation of this result is that each of these mutants was unable to interact with one or more of the essential general transcription factors required for preinitiation complex assembly. To test the role of TFIIB in activated transcription, we derived more discrete mutants. Based on the results of Fig. 2, we reasoned that the integrity of the putative basic amphipathic α -helix in the first repeat was essential for the VP16-TFIIB interaction. Figure 3 shows the analysis of four double substitution mutants of basic residues within the putative amphipathic α -helix. Substitution of the two basic amino acids at positions 185 and 189, or 196 and 200, to neutral residues (R185L/K189L and K196L/K200L) significantly reduced binding of GAL4-VP16. The most dramatic effects, however, were observed with two mutants in which basic amino acids were changed to acidic residues: there was no detectable binding of GAL4-VP16 to either R185E/R193E or K189E/K200E. These results confirm that the basic residues of the putative amphipathic α -helix are critical for interaction with VP16.

Because the TFIIB mutants R185E/R193E and K189E/K200E were the most severely compromised for the VP16-TFIIB interaction, they were selected for transcription experiments. Figure 4 analyses the ability of these TFIIB mutants to support transcription in a HeLa cell nuclear extract depleted of endogenous TFIIB by passage over a GST-VP16 column^{1,2}. The assays were done using a well characterized adenovirus E4 promoter derivative containing five GAL4 binding sites upstream of the TATA box (see refs 1, 2). Figure 4a shows, as expected, that in a TFIIB-depleted nuclear extract basal transcription was severely compromised, and could be restored by addition of recombinant wild-type TFIIB. Significantly, both the R185E/R193E and K189E/K200E substitution mutants could restore basal transcription to 100% and 80%, respectively, of the wild-type level (for quantitation, see Fig. 4c). We note that at the highest protein concentration, both wild-type TFIIB and the derivatives modestly inhibited transcription, a result consistent with a previous study⁶. The R185E/R193E and K189E/

K189E/K200E substitution mutants also supported basal transcription of the adenovirus major late promoter in a system reconstituted with purified components (data not shown).

Next, we assayed the ability of these two TFIIB derivatives to support transcription directed by acidic activators. In Fig. 4b, a TFIIB-depleted nuclear extract was supplemented with either wild-type TFIIB, or one of the TFIIB substitution mutants, and transcription analysed in the presence of either of two acidic activators, GAL4-VP16 (upper panel) or GAL4-AH (lower panel). As expected, the transcriptional activity of a TFIIB-depleted nuclear extract was undetectable, and addition of wild-type TFIIB restored activated transcription to a level comparable to that observed in the crude nuclear extract. In contrast, neither of the TFIIB derivatives supported activated transcription even at the highest concentration of TFIIB tested. Rather, the two TFIIB mutants gave rise to a low level of transcription, most evident in the experiment with GAL4-AH (lower panel), which approximates the basal level in the absence of an activator.

The data shown in Fig. 4a and b were quantitated and summarized in a bar graph in Fig. 4c. We conclude that the TFIIB derivatives R185E/R193E and K189E/K200E can support basal transcription but are severely, and perhaps completely, defective in supporting transcription directed by two unrelated acidic activation regions.

Our results connect our previous complex assembly¹ and protein-protein interaction^{1,2} assays with transcription; without this link the interaction between an acidic activator and TFIIB^{1,2}, or the effect of an acidic activator on TFIIB assembly¹, might not be directly relevant to transcriptional activation.

There are now several independent lines of evidence indicating that TFIIB is a pivotal component in the mechanism by which an acidic activator functions. First, in assays that monitor preinitiation complex assembly, TFIIB binding can be a limiting step, and this step can be enhanced by an acidic activator¹. Similarly, in kinetic assays using a different promoter and an unrelated activator, the TFIIB binding step was found to be slow

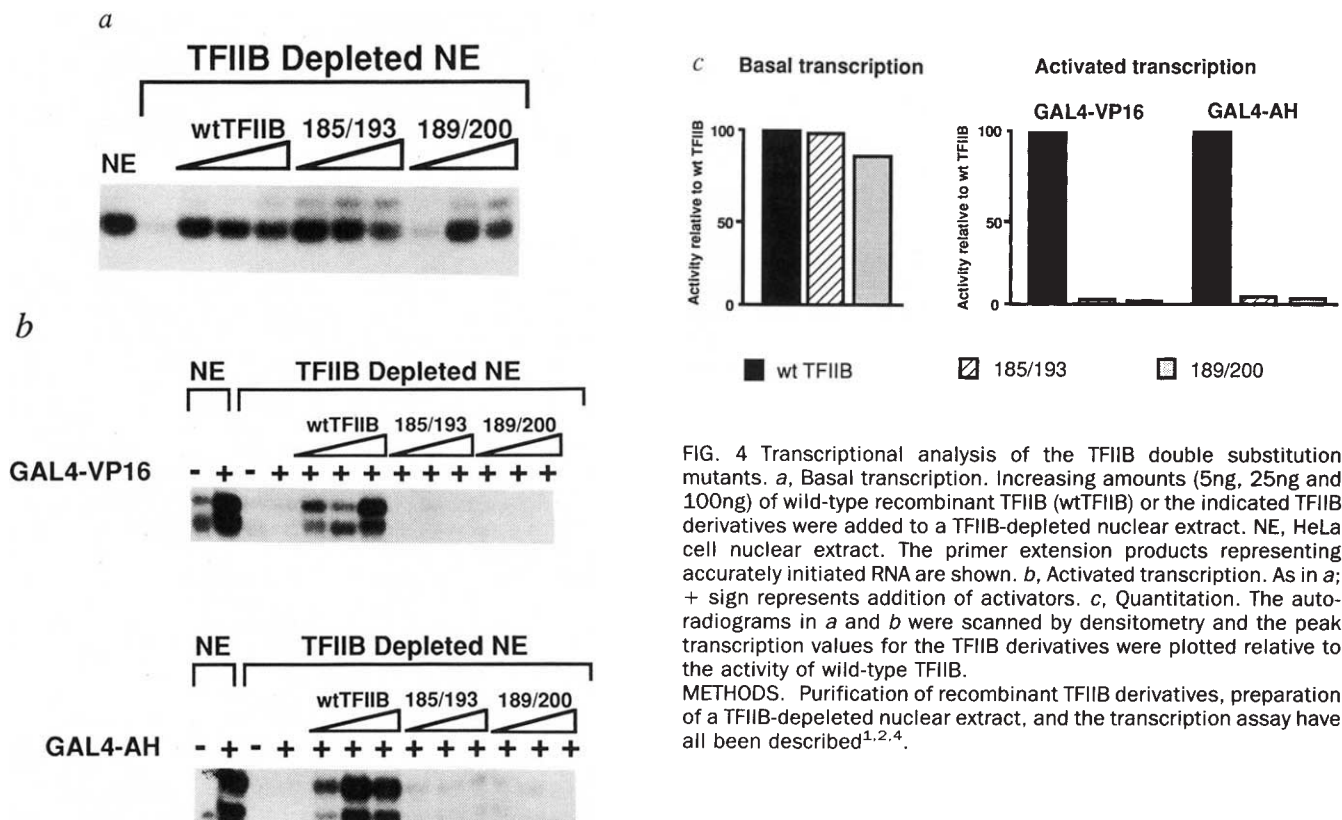


FIG. 4 Transcriptional analysis of the TFIIB double substitution mutants. *a*, Basal transcription. Increasing amounts (5ng, 25ng and 100ng) of wild-type recombinant TFIIB (wtTFIIB) or the indicated TFIIB derivatives were added to a TFIIB-depleted nuclear extract. NE, HeLa cell nuclear extract. The primer extension products representing accurately initiated RNA are shown. *b*, Activated transcription. As in *a*; + sign represents addition of activators. *c*, Quantitation. The autoradiograms in *a* and *b* were scanned by densitometry and the peak transcription values for the TFIIB derivatives were plotted relative to the activity of wild-type TFIIB.

METHODS. Purification of recombinant TFIIB derivatives, preparation of a TFIIB-depleted nuclear extract, and the transcription assay have all been described^{1,2,4}.

and could be accelerated by an activator⁷. Second, both HeLa TFIIB^{1,2} and recombinant TFIIB² interact with an acidic activation region. Third, as shown here, TFIIB mutants unable to interact with the VP16 acidic activation region are specifically defective in supporting transcription directed by acidic activators. Conversely, a VP16 single amino-acid-substitution mutant, which cannot activate transcription⁸, is correspondingly defective for interaction with TFIIB^{1,2}.

Although we have shown that the VP16-TFIIB interaction is necessary for activated transcription, our results are not inconsistent with the observation that factors other than TFIIB, such as the TATA-box-binding protein (TBP), may also directly interact with an acidic activator^{9,10}. A set of transcription components, referred to as adaptors¹¹, coactivators¹² or mediators^{13,14}, are required for activated but not basal transcrip-

tion (reviewed in refs 15, 16). Again, our results are not inconsistent with this possibility; such coactivator activities may also function through direct contacts with the activator, or act at another step (s) in the transcription activation pathway.

These considerations raise the possibility that activators may function through multiple targets and at several steps. To help explain how eukaryotic activators stimulate transcription synergistically (reviewed in ref. 17), we have suggested that the multiple promoter-bound activators may contact more than one general transcription component^{1,2}. The possibility that activators function at several steps would also explain why raising the concentration of a single general transcription factor does not overcome the requirement for an activator (see, for example, refs 6, 18). □

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Functional dissection of TFIIB domains required for TFIIB-TFIID-promoter complex formation and basal transcription activity

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THE protein TFIIB is a general transcription initiation factor¹ that interacts with a promoter complex (D-DNA) containing the TATA-binding subunit (TFIID_T, or TBP) of TFIID to facilitate subsequent interaction with RNA polymerase II (ref. 2) through the associated TFIIF (ref. 3). The potential bridging function^{2,4} of TFIIB raises the possibility of two structural domains and emphasizes the importance of TFIIB structure-function studies for a further understanding of preinitiation complex assembly and function¹. Here we show that human TFIIB (refs 5,6) is comprised of functionally distinct N- and C-terminal domains. The C-terminal domain, containing the direct repeats and associated basic regions, is necessary and sufficient for interaction with the D-DNA complex. By contrast, the N-terminal domain that is dispensable for formation of the TFIID-TFIIB-promoter (D-B-DNA) complex is required for subsequent events leading to basal transcription initiation. On the basis of these results, we discuss structural and functional similarities between TFIIB and TFIID_T, which have similar structural organization and motifs⁵.

Structural features important for the two fundamental activi-

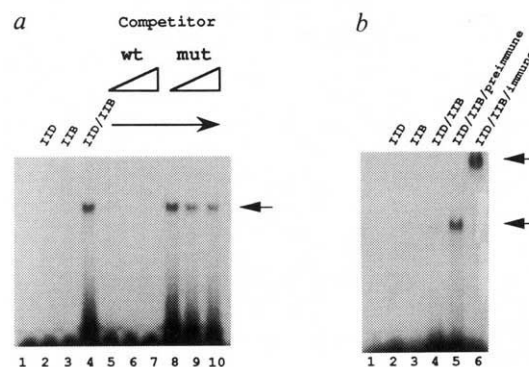


FIG.1 TFIIB interacts cooperatively with TFIID_T on the promoter. *a*, Specificity of D-B DNA complex formation. Electrophoretic mobility shift assay (EMSA) reactions contained the AdML promoter probe (positions -40 to +10) and, as indicated, either TFIID_T, TFIIB, or both, and, as indicated, increasing amounts (25, 50 and 100 molar excess) of competitor oligonucleotides (31 bp, position -45 to -15 of the AdML promoter) containing wild-type (wt; TATAAAA) or mutant (mut; TAGAGAA) TATA sequence. *b*, Presence of TFIIB in D-B DNA complexes. As indicated, EMSA assays (as in *a*) contained TFIID_T (10 ng), TFIIB (10 ng), or both, and either anti-TFIIB antiserum or preimmune serum. The lower arrow indicates the D-B DNA complex and the upper arrow indicates the anti-TFIIB supershifted complex. The enhancement of D-B DNA complex formation was reproducibly seen when serum was added (lanes 4 and 5).

METHODS. Recombinant human TFIID_T was purified as described²⁴. TFIIB was purified as in Fig. 2. EMSA reactions contained 6 ng ml⁻¹ ³²P-labelled major late promoter fragments, the indicated amount of TFIIB and TFIID_T in 12 mM Tris-HCl, pH 7.9, 20 mM HEPES-KOH, pH 8.4, 60 mM KCl, 7.5 mM MgCl₂, 12% glycerol, 0.12 mM EDTA, 6mM β-mercaptoethanol, 20 mM DTT, 0.12 mg ml⁻¹ BSA and 10 μg ml⁻¹ poly dGdC. Reactions were incubated at 30 °C for 40 min and the products were resolved in 1 X TBE in 4% polyacrylamide gels (60:1). Antibodies against TFIIB were obtained from a rabbit injected with purified Δ3-55. Both immune (2 μl) and preimmune serum (2 μl) were added to the reactions after 40 min incubation; reactions were further incubated for 10 min.

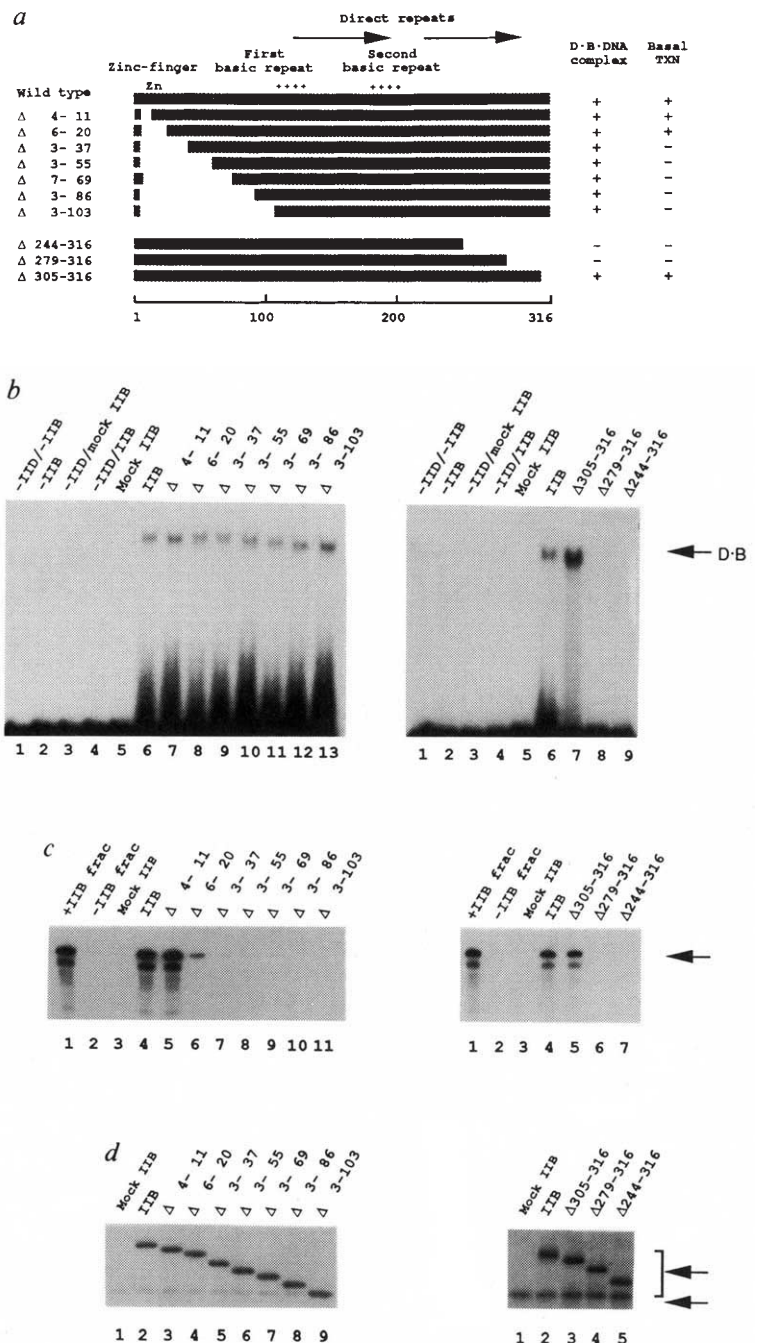
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ties of TFIIB, D·B·DNA complex formation and basal transcription, were analysed using a systematic series of deletion mutants. Formation of the D·B·DNA complex was monitored by electrophoretic mobility shift assay with purified human TFIID τ and TFIIB. These analyses were done in the absence of TFIIA (which facilitates TFIID binding to DNA)^{2,7} as it is not essential for formation of D·B·DNA (and higher-order) complexes⁸, and in order to evaluate primary interactions between TFIID τ and TFIIB on the DNA. Under the present conditions, TFIID τ and TFIIB interact cooperatively (Fig. 1a,b) and in a dose-dependent fashion (data not shown) to form a D·B·DNA complex. The specificity of the complex and the involvement of both TFIID τ and TFIIB are demonstrated further by the sensitivity of the complex to competing oligonuc-

leotides containing wild-type but not mutant TATA sequences (Fig. 1a), and by the reduced mobility (supershift) with anti-TFIIB, but not preimmune, serum (Fig. 1b). The presence of TFIIB is further indicated by variations in mobility with differently truncated N-terminal mutants (Fig. 2b, below).

To localize the region(s) of TFIIB important for D·B·DNA complex formation versus basal transcription, a series of N-terminal, C-terminal and short internal deletion mutants were expressed in and purified from bacteria (Figs 2d, 3d) and analysed for complex formation (Figs 2b, 3b) and transcription (Figs 2c, 3c). D·B·DNA complex formation was largely unaffected by N-terminal deletions extending to a position (103) 20 residues from the start of the first direct repeat, or by a C-terminal deletion extending to a position (305) close to the

FIG. 2 Effect of N- and C-terminal deletions of TFIIB on D·B·DNA complex formation and basal transcription (TXN). a, Summary of mutant TFIIB proteins and functional analyses. The left column summarizes the mutants with (inclusive) deleted residues indicated. The right column summarizes qualitatively the results of EMSA and basal transcription assays in b and c. Upper diagram indicates the approximate positions of the zinc-finger (residues 15 to 37), the first (105 to 127) and second (175 to 200) basic-residue repeats, and the first (122 to 198) and second (216 to 292) direct repeats. b, D·B·DNA complex formation with N- and C-terminal deletion mutants. Reactions in lanes 1–4 (left and right panels) contained the AdML probe and either no protein (lane 1), 10 ng TFIID τ (lane 2), 10 ng mock-purified TFIIB protein (lane 3) or 10 ng TFIIB (lane 4). Reactions in remaining lanes (left and right panels) contained the same AdML probe, 10 ng TFIID τ , and 10 ng of the mock TFIIB, wild-type TFIIB or mutant TFIIB, as indicated. c, *In vitro* transcription assays with N- and C-terminal deletion mutants. Reactions contained AdML core promoter template, partially purified TFIID and TFIIE/F, RNA polymerase II (ref. 25) and 10 ng TFIIB preparations. Arrow indicates the position of the specifically initiated transcript. d, SDS-PAGE analysis of the mutant TFIIB proteins; upper arrow indicates the positions of the mutant TFIIB proteins; lower arrow indicates a contaminating *E. coli* protein that specifically binds to Ni-agarose resin. METHODS. Deletion mutants were made according to ref. 26, confirmed by DNA sequencing and subcloned into 6HisT-pET11d to contain the 20-amino-acid histidine tag²⁷. Expression and purification of mutants were essentially as described¹⁰. For C-terminal and internal deletion mutants, proteins were denatured in 6 M guanidine-HCl to solubilize the mutants. The eluate was dialysed against 2 M guanidine-HCl for 1 hour and then against 1 M guanidine-HCl overnight. To adjust the amount of each mutant TFIIB, it was diluted with the nickel column eluate prepared identically from *E. coli* harbouring 6His-pET11d without any insert (d, lane 1). EMSAs were as described in Fig. 1 legend; 10 ng TFIIB mutant and TFIID τ were used. *In vitro* transcription has been described²⁸.



end of the second direct repeat (Fig. 2b, left panel). Although further N-terminal deletions could not be analysed because of mutant protein instability in the expression system, C-terminal deletions extending to positions (244, 279) within the second direct repeat abolished complex formation (Fig. 2b, right panels, lanes 8, 9). Further analyses with internal deletions confirmed these results (Fig. 3b). All deletions within the direct repeat region, as well as deletion of 16 residues (Δ 106–121)

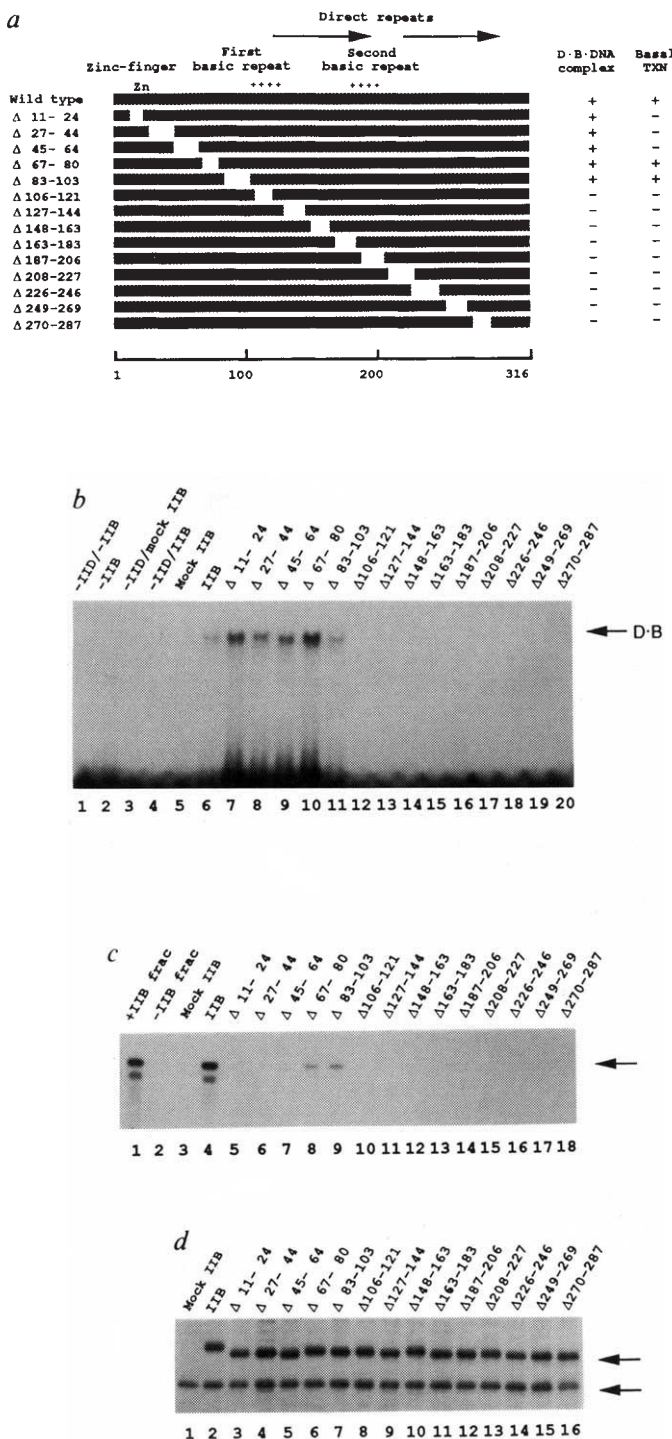


FIG. 3 Effect of internal deletions of TFIIB on D-B-DNA complex formation and basal transcription activity (TXN). All experimental procedures and explanations of the panels are the same as in Fig. 2.

immediately preceding the first direct repeat, eliminated complex formation, whereas all but one internal deletion mutant in the region preceding the first direct repeat maintained the capability for complex formation (Fig. 3b). These results (summarized in Figs 2a, 3a) implicate the highly conserved direct repeat domain, as well as the short region containing the first basic repeat^{5,6,9,10}, in interactions leading to D-B-DNA complex formation. In view of the cooperative interactions of TFIIB and TFIID τ on the promoter and the lack of demonstrable DNA binding of TFIIB alone, it is presumed that complex formation involves, at least in part, direct interactions between TFIIB and TFIID τ .

To determine the probability of separable functional domains in TFIIB, the various deletion mutants were also analysed for their ability to support basal transcription from the adenovirus major late (AdML) core promoter in conjunction with other general factors. As shown in Figs 2c and 3c and summarized in Figs 2a and 3a, all mutations that eliminated D-B-DNA complex formation also eliminated basal transcription, consistent with the proposed role of TFIIB. But in contrast to the results for D-B-DNA complex formation, most of the deletion mutants in the region N-terminal to the first direct repeat eliminated or markedly reduced transcription. Those residues in this region that were completely dispensable included amino acids 4–11 (Fig. 2c, lane 5) and those whose removal reduced, but did not eliminate, transcription included amino acids 6–20, 67–80 and 83–103 (Fig. 2c, lane 6; Fig. 3, lanes 8, 9). Thus, although these latter residues may be important for overall efficiency of transcription, they are not absolutely essential, whereas residues 21–66 are both essential and uniquely required for transcription versus D-B-DNA complex formation. The sequence of this region is well conserved in TFIIB from other species and in BRFI (ref. 11)/(PCF4 (ref. 12)/TDS4 (ref. 13), a TFIIB-related factor involved in RNA polymerase III transcription. Although this region, in principle, could function at a number of later steps in transcription, it is probably required for RNA polymerase II recruitment (via TFIIF) to the complex (S. Malik and R.G.R., unpublished results). The region between residues 6 and 20 is of particular interest because it contains part of a putative zinc-finger structure⁴ which could contribute to transcription efficiency by protein-protein interactions^{14,15}.

These structure-function studies have revealed intriguing functional similarities between TFIID τ and TFIIB, previously noted⁵ (Fig. 4) to be similar with respect to the overall structural organization of C-terminal direct repeats, basic residue repeats and presumptive sigma homologies^{4,6,9,10}. Just as the TFIID τ direct repeats are required for forming a TFIID τ -promoter complex^{16–18} (and for subsequent steps), so are the TFIIB direct repeats necessary for formation of a TFIIB- and TFIID τ -containing promoter complex. Although protein-protein interactions may be the main determinants for recruitment of TFIIB to the promoter, in contrast to the situation for TFIID τ , DNA- sequence-independent interactions of TFIIB with DNA may also be important. At the same time, DNA-sequence-independent interactions of TFIID τ may also be expected, especially in TATA-less promoters. In terms of tertiary structure, X-ray crystallographic studies of TFIID τ have revealed intramolecular structures, comprised of the direct repeats and short flanking regions, with striking twofold symmetry¹⁹. Given the structural and functional similarities between the C-terminal domains of TFIIB and TFIID τ , it would not be surprising if the C-terminal domain of TFIIB also had an intramolecular symmetry important for interactions with the TFIID τ -promoter complex.

Despite these similarities, TFIIB also shows some important distinctions from TFIID τ , especially with respect to the N-terminal regions preceding the direct repeats. This region is variable in length and sequence in different TFIID τ species^{20–22} and is not necessary either for promoter recognition or basal transcription⁷. In contrast, the TFIIB N terminus is highly

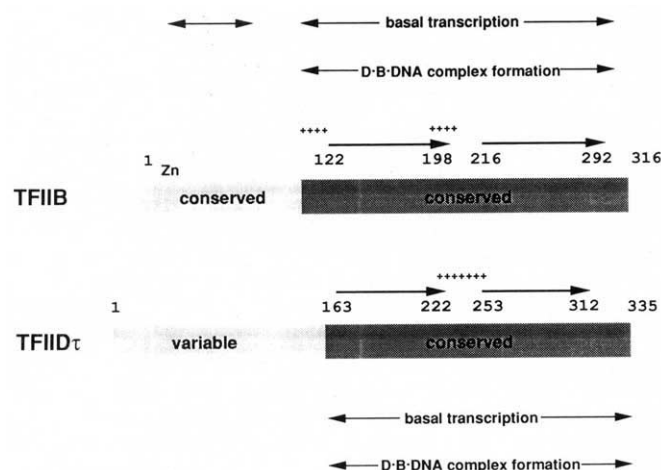


FIG. 4 Structural and functional comparisons of TFIIB and TFIIDr. The scheme shows the overall structure of TFIIB and TFIIDr. The position of structural motifs^{4-6,9,10,16} are shown. Domains necessary for D-B-DNA complex formation and basal transcription activities are shown.

conserved in evolution in both length and sequence^{4-6,9,10,23}, and, although it is unnecessary for stable binding to TFIIDr-promoter complexes, it is required for basal transcription. The fact that TFIIB serves at least in part as a bridge between promoter-bound TFIIDr and RNA polymerase II/TFIIF has most probably imposed evolutionary constraints on the N terminus as well as the C terminus. Further studies on the structure and function of TFIIB should help elaborate features important for both transcription initiation and its regulation. □

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From infrared to ultraviolet

New products in the field of spectroscopy include a fluorescence spectrophotometer for routine assays, ultraviolet/visible range systems, and ultra micro cells for 0.5 µl volume.

THE SX.17MV BioSequential **stopped-flow spectrofluorometer** from Applied Photophysics has been designed for chemical, biochemical and molecular biological reaction mechanisms (*Reader Service No. 100*). The company states that this new instrument features sub-millisecond dead time, advanced circular dichroism detection that is accurate to better than 0.005°, absorption detection accurate to 0.001 AU and f/0.4 fluorescence collection efficiency. It offers the operator the ability to collect fast time-resolved spectra (80,000 per second) and incorporates a multiparametric analysis package (including numerical integration). Specially designed low inertia drive rams combine with a computer-optimized flow circuit to provide high sample flow rates and optimum mixing. (6 to 8 M urea or guanidinium chloride against aqueous buffer is achieved.) Applied Photophysics says that accurate variable ratio stopped and sequential flow allow for all protein folding, unfolding and re-folding experiments.

The Personal Spectrometer II, a new portable **diode-array-based spectroradiometer** covering the spectral region from 350 nm to 1,050 nm, is manufactured by Analytical Spectral Devices and distributed by Glen Spectra (*Reader Service No. 101*). Utilizing a plasma-coupled 512-element photodiode array with fibre-optic input, the Personal Spectrometer II can acquire up to five spectra per second with 1.4-nm sampling and a spectral resolution of better than 3 nm. Weighing in at 3.3 kg, it has a 1-MB nonvolatile RAM cartridge allowing onboard storage of more than 600 spectra in addition to a 1.44 MB 3.5-inch floppy disk drive allowing storage of a further 900 spectra per disk. Rechargeable batteries allow over 3 h of continuous use in the field and an onboard PC-compatible computer allows information to be displayed as NDVI, PPFD, chromaticity coordinates and SPOT and TM bands with a radiometric calibration function to display spectra in absolute irradiance or radiance units as well as reflectance units. Accessories for the Personal Spectrometer II include a laser pointing device, a remote cosine collector, a satellite global positioning system, a radiometric calibrator and reflectance reference panels.

Shimadzu Scientific Instruments introduces the RF-1501 **fluorescence spectrophotometer** (*Reader Service No. 102*). The instrument is designed to offer high sensitivity (300 S/N at 350 nm excitation, bandwidth 10 nm, 2.0 second time constant),

speed (about 30,000 nm min⁻¹ slew speed) and a cost of about US\$12,500. Although intended for routine fluorescence assays, the RF-1501 also makes fluorescence research and method development easier and more accurate, Shimadzu says. It has an optimum wavelength search feature that automatically detects the excitation and emission peaks by scanning the whole wavelength range of the sample. Other features are its single sample holder, which allows the user to perform small sample applications, and its interchangeable program packs to make it easy to move from one application to the next.

Baird Analytical, a division of Imo Industries, announces the availability of its Spectrovac 2000 **arc/spark optical emission spectrometer** (*Reader Service No. 103*). The instrument can be used to measure all elements in their ferrous and/or non-ferrous alloys. The Spectrovac 2000 is a one-metre vacuum spectrometer system with a sixty-channel capacity for the measurement of all elements simultaneously. With its optional dual-stand sample, dissimilar materials can be analysed without cross-contamination, Baird says. An eight-page brochure on the system illustrates the advantages of utilizing a d.c. arc source, in addition to the illumination system which uses fibre optics. The system's software, customer support and instrument specifications are also reviewed.

■ Ultraviolet/visible range

Hewlett-Packard introduces the HP 8452Win **diode-array ultraviolet/visible spectroscopy system**, which runs under Microsoft Windows software (*Reader Service No. 104*). The HP 8452Win system has an extended feature set and is designed for two main analytical tasks: method development and research requiring spectral manipulation, data evaluation and quantification, and routine testing applications



Hewlett-Packard's new UV/visible system runs under Windows.

that require automation and compliance with Good Laboratory Practices. Up to three confirmation analysis methods can be used in addition to the main analytical method to check the identity of samples and to detect impurities and other sources of error. Full spectral data are always available. The statistical tools and method development tasks include: correlation coefficient and other statistical values, an evaluation of standards and wavelength optimization. The system is controlled by a UV/visible HP ChemStation that provides full data management, system control and networking. To simplify operation, the software adheres to industry standards for Microsoft Windows, including screen displays, data communication and networking. Analyses can be automated using a sipper autosampler, multicell transport and a multiple-choice selection of automation options.

A **head-on photomultiplier tube** is now available as an accessory for the U-3000 and U-3300 ultraviolet/visible spectrophotometers from Hitachi Scientific Instruments (*Reader Service No. 105*). The accessory is



Hitachi's head-on photomultiplier extends the wavelength range to 190 nm.

designed to ensure maximum sensitivity for transmittance and reflectance measurements of diffuse solid and liquid samples, particularly in the UV region of the spectrum. The head-on photomultiplier tube is used as an alternative to an integrating sphere when measurements in the UV are of particular importance. Hitachi says that the sensitivity of an integrating sphere decreases significantly below 240 nm, but the head-on photomultiplier extends the wavelength range to 190 nm. The standard effective wavelength range is 190–800 nm, with a baseline flatness specification of ±0.5 per cent T. In addition, a red-sensitive version is also available, allowing measurements to 900 nm. The tube can be easily substituted for the standard integrating sphere detector,

and accommodates samples up to 200 mm in length. The U-3000 series of spectrophotometers equipped with the head-on photomultiplier are used in several applications, particularly for the measurement of highly turbid samples such as cytochromes in yeast. Even though the baseline level is around 2.5 absorbance units, the large dynamic range of the U-3000 enables spectra up to 5 absorbance units to be recorded without clipping. The head-on photomultiplier tube can also be used as a detector for the analysis of quartz and calcite where absorption characteristics between 190 and 210 nm are of particular interest. The accessory is also suitable for use in conjugation with 12 degree and 45 degree specular reflectance attachments.

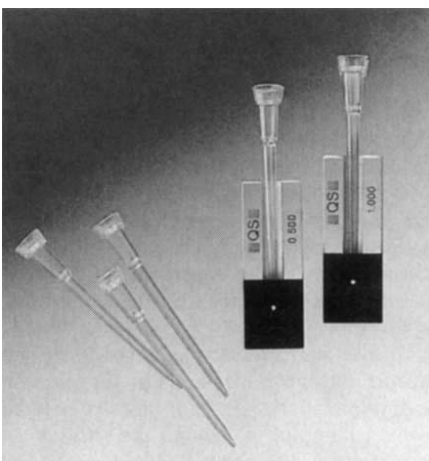
Milton Roy announces the introduction of the latest instrument in its line of Spectronic spectrophotometers — the Genesys 5 **spectrophotometer** (Reader Service No. 106). This ultraviolet/visible spectrophotometer provides a 5-nm slit width over the 200–1,000-nm wavelength range, and contains an 8-position cell holder for the precise, automatic positioning of sample cells. The built-in graphic LCD screen provides the operator with pertinent details of the test being performed such as application program type, analytical wavelength, sample identification, test name and test results. Through a series of pictograms, lamp, accessory and memory card status is also presented on the screen. Results can be sent to the optional internal printer or to various external devices via the built-in RS-232-C or Centronics-compatible ports. The instrument is capable of performing simple photometric measurements in absorbance, percentage transmittance and concentration. With optional application and memory SoftCards, the user is able to set and store parameters for survey scan, linear or nonlinear standard curves, and kinetics/time scan programs, as well as store and manipulate data (test results). A file manager is also available for copying and general maintenance of test or data files.

Perkin-Elmer's new Lambda Bio ultraviolet/visible spectrometer enables researchers to run more than 50 pre-defined methods with a single keystroke, providing fast results for **DNA and protein characterization** (Reader Service No. 107). Without the need for programming or method development, Perkin-Elmer says that users can run methods for nucleic acid, protein, kinetic and general quantitative and qualitative UV/visible analyses. Designed for easy operation, the Lambda Bio can also run methods developed for specific application needs. A space-saving work tray facilitates sample preparation and a built-in printer provides documentation of results. Accessories for the instrument include auto cell changers and sippers, and temperature-controlled cell holders (water-thermostatted or Peltier).

■ Accessories

The new 1993 **spectrophotometer cell catalogue** from NSG Precision Cells lists over 300 different types of cells (Reader Service No. 108). Most cells are available in a choice of optical glass, ultraviolet quartz, infrared quartz or ES quartz. The fully illustrated catalogue features standard cells and special cells that include anaerobic cells, micro cells, water-jacketed cells and dye laser cells. Accessories such as cell washers and mixers are also included.

The new series of **ultra micro cells** from Hellma has been designed to facilitate the spectrophotometric analysis of minute volumes (Reader Service No. 109). The series comprises four cells: 5- μ l volume/10-mm light path, 2.5- μ l volume/5-mm light path,



Hellma's ultra-micro cells with minute volumes.

5- μ l volume/1-mm light path and 0.5- μ l volume/0.1-mm light path. Hellma says that these cells can be filled and emptied with even the smallest volumes through the filling/emptying ports using a pipette and suitable tips when the sample volume is just slightly larger than the light path chamber volume. The company is also supplying **standard microplates manufactured from quartz**. Besides being useful in processes that involve the use of chemicals such as acids, alkalis and solvents, the plates allow measurements to be taken at wavelengths in the ultraviolet region of the spectrum. This should be of use in research involving inverted microscopes.

■ FT-IR range

A broad spectral range is the hallmark of the new IFS 55 **FT-IR spectrometer** from Bruker Instruments (Reader Service No. 110). Using different sources as well as several beamsplitters and detectors, the instrument can optionally span the range from 50 to 15,000 cm^{-1} . The spectrometer can house two internal detectors as well as up to three different sources; all switching between detectors and sources is under compu-

ter control. The IFS 55 offers a resolution of 0.5 cm^{-1} , with an option for an apodized resolution of better than 0.2 cm^{-1} . The scanner can achieve up to 20 scans per second at 8 cm^{-1} resolution, Bruker says. The spectrometer is supplied as a sealed and desiccated system, and the base configuration requires no cooling water or compressed air for the frictionless mechanical scanner. The large sample compartment can be isolated from the rest of the spectrometer and optionally purged. In addition to a full palette of spectral ranges, the IFS 55 features up to six different beam ports. With one as an exit port and a second as an emission port, the IFS 55 allows each of the remaining four ports to be configured either as an exit or as an entrance (emission) port. This, Bruker says, opens up many possibilities for sample configurations. Several sampling accessories are available for the IFS 55, including an IR-microscope, the FRA 106 FT-Raman accessory, chromatographic couplings, as well as near-IR fibre-optic probes. The system is supplied with a PC-486, 50 MHz processor that controls all instrument functions. Software packages are available for applications that include library search, partial-least-squares quantitative analysis and time-resolved spectroscopy.

The new Magna-IR Series **FT-IR spectrometers** from Nicolet are designed to combine spectroscopic sampling capability with a Microsoft Windows operation system (Reader Service No. 111). Two systems are available: the 550 mid-IR system and the more advanced 750 system with an extended wavelength capability. The 550 system can be upgraded to the 750. All Magna-IR components are pre-aligned. The precision-cast Stabilizer baseplate combines tight surface tolerances with pinned-in-place, diamond-turned mirrors for consistent optical alignment. Nicolet says that the Ever-Glo mid-infrared source is thermally stable. The DSP-driven interferometer, Vectra, controls all scan parameters. OMNIC software incorporates the Windows-based graphical user interface to collect data with real-time spectral display by using pull-down menus. Software functions like deconvolution and spectral subtraction are performed in the same way. When needed, OMNIC multitasks with other Windows-compatible software, continuing analysis and reporting Magna-IR's scan status while the user works in other applications. The company says that Magna-IR has options for better than 0.1 cm^{-1} resolution and spectral range options that cover the near-, mid- and far-infrared regions. Monoflect dual-detector optics allow simultaneous mounting of two onboard detectors, and a dual external beam option offers added flexibility.

■ Atomic absorption spectroscopy

Cole Parmer has introduced the new **atomic absorption spectrophotometer**, which should be of interest to researchers in the

PRODUCT REVIEW

fields of environmental testing, toxicology and quality control (*Reader Service No. 112*). The spectrophotometer allows the operator to measure as many as 70 different elements. Slit width is adjustable and complies with many Environmental Protection Agency methods. The digital readout displays absorption, concentration, emission values, lamp readings and photomultiplier voltage. Other design features include control and selector valves for oxidant and auxiliary gases; a fuel control valve; dual flowmeters that show fuel and oxidant rates; easy-to-use controls for burner position; front-panel controls for calibration, display and lamp operations; and push-button control of integration, read/decimal, damping and autozero functions. Cole-Parmer says that integrator mode averages 1,900 readings over 7 s for increased accuracy, damping mode reduces noise, and curve correction mode adds a second calibration point in nonlinear region of absorption/concentration curve.

The Unicam 929 is a new member of the SOLAAR series of **spectrometers** that are designed to bridge the gap between low-cost

manual atomic absorption and fully automatic multielement atomic absorption (*Reader Service No. 113*). The modular design enables the addition of double-beam Stockdale optics, quad lamp turret, automatic gas control and a high-energy background correction facility. The SOLAAR 929 system is available as a standalone, locally-controlled spectrometer or, by the addition of an IBM-compatible data station running Microsoft Windows software, the full power and flexibility of the 929 is realized, Unicam says. SOLAAR 929 local control allows automated flame or vapour analysis to be performed with confidence using an interface that utilizes a minimum key configuration keypad with integrated liquid crystal display. SOLAAR software running under Microsoft Windows 3.1 is offered at two levels: routine software provides automatic flame and vapour analysis with the emphasis on routine analysis, and the advanced software provides more extensive options for sampling, data processing and analysis in all atomic absorption techniques. The SOLAAR 929 provides either a dedicated furnace option or the ability to add a GF90 graphite furnace to a flame system. Automatic sample preparation and presentation is comprehensive using precision autosamplers, segmented flow injection/vapour systems and the furnace, Autoprobe.

Varian has introduced new versions of its **atomic absorption spectrometers**, which feature advanced software that is designed to help laboratories ensure the validity of results (*Reader Service No. 114*). The SpectraAA-300/400 Plus spectrometers include extensive sample validation and tracking software. Accessed via a standalone PC, the software provides a choice of zero and non-zero calibration curve fits, and the ability to mask erroneous standards, which automatically overcomes many standard preparation errors for enhanced productiv-

ity. The systems include upper and lower control limits, spike recoveries, and duplicate calculations, so that operators can better track the validity of analytical results. Long-term archiving with a database engine provides search and retrieve facilities and graphical trend analysis, for data access and analysis. The SpectraAA-250 Plus features double-beam optics for enhanced performance and long-term stability. A four-lamp motorized turret, with fully automatic wavelength and slit selection, is designed to simplify set-up and operation and speed sample determinations. The instrument features a PC-controlled Hammer gas control system. The system is also compatible with new accessories for graphite furnace and vapour generation atomic absorption.

The new Analyte MEFF-16 **atomic absorption instrument**, which, when fitted with an Accutrace furnace atomizer, can be used to determine the concentrations of all the trace elements in an individual sample in rapid sequence (*Reader Service No. 115*). Each sample is completed before the next one is begun. As the elements are determined sequentially rather than simultaneously, Analyte says that each can be measured under its optimum furnace conditions. The sample is deposited into the furnace in the form of a vapour by the Accumist nebulizer. Such deposition has the benefits of reducing chemical interferences, and can also regulate analytical sensitivity as required by adjusting the deposition time. In addition, the Accutrace furnace uses side-heated graphite tubes. Such tubes, in contrast to the more conventional end-heated devices, are heated more uniformly along their length, again with the effect of reducing analytical interferences. In a single sample, the furnace can use different matrix modifiers to determine individual elements. In addition to 120 samples, the autosampler can contain six standards, four matrix modifiers and a blank solution.

Shimadzu, with distribution by V. A. Howe, has introduced a **microvolume flame analysis system** with its new AA-6501 Series atomic absorption spectrophotometers which uses a typical 60- μ l sample volume (*Reader Service No. 116*). This technique can be fully automated with the use of the ASC6000 'semi-intelligent' preparation station. Standard curves can be produced from a single stock standard, essential matrix modifiers added automatically and analysis performed on up to 60 samples. This microvolume technique is also useful when sample concentration is necessary. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.

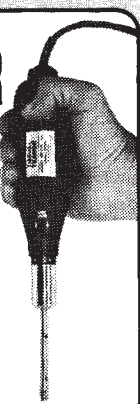
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Varian's SpectraAA-300/400 Plus atomic absorption spectrometer.

Working in a wider Europe

Alison Abbott and Declan Butler compare and contrast the cases of Germany and France. What is it like to work in these two very different countries?

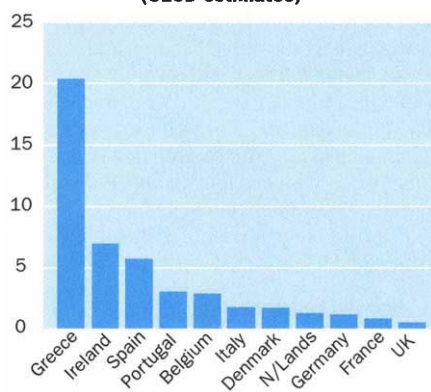
THERE are more than 30 countries in Europe (outside the former Soviet Union) and the number seems to increase daily. There may be twice as many languages, and innumerable different cultures and histories. This mosaic is both an attraction and a disincentive to foreign scientists both from within and outside the continent.

Mobility does not come easily to Europeans, not least because of language difficulties. English may be the international language of science, but a scientist also needs to be able to buy food or get a haircut. Scientists from outside Europe may find the language problem even more daunting. But this has not stopped thousands per year testing the water somewhere on the continent.

And then there are scientists from central and eastern Europe who in the past few years have regained their freedom to travel, but not the financial wherewithal. They are turning in droves to the many assistance schemes to help them gain experience in a western European lab.

EC grants. Scientists within the twelve countries of the European Community (EC) are being encouraged by the Ecu488-million (US\$710-million) EC Human Capital and Mobility scheme to go and work in a neighbouring EC country. The scheme was set up last year as a political tool to increase human research resources in a united Europe. It offers two-year grants to individuals, and support for the host institutes. The scheme has been criticized by scientists for the opacity of the application procedure and the chaos of the internal organization, but

Applicants for Human Capital and Mobility grants per thousand national R&D population (OECD estimates)



new EC research minister Antonio Ruberti has promised to make life easier for applicants by simplifying forms and establishing fixed (rather than short-notice) deadlines.

Applications are assessed several times a year. Preliminary statistics are starting to give interesting pointers about who is going where (see table). Poorer EC countries have shown the most interest in the scheme (in relation to the size of the research and development population) with Greece submitting 20.18 applications per thousand and Ireland 6.8 (see graph). The four biggest economies in Europe showed less relative interest in moving around the continent: Germany, 1.06; Italy, 1.66; France, 0.86; and UK only 0.48. The poor showing of France and UK may be related to the excellence of home facilities: these two countries also received the most applications (313 and 270 respectively). But this is unlikely to be the

true answer. It would not explain, for example, why rich Germany attracted only 80 applications. The figures represent a complex brew of language, cultural, political and economic factors whose analysis will no doubt fuel many a sociology thesis.

France

"Broken English," joked CNRS director general François Kourilsky as he chatted with a group of foreign visitors recently, "is the international language of science". He may soon remark that broken French is the internal language of CNRS (Centre National de la Recherche Scientifique). Almost one in ten of its 11,417 life-tenure researchers is now non-French, and their numbers are growing. Last year, foreign scientists accounted for almost one quarter of all CNRS recruits, and around half those at the senior grade of *directeur de recherche première classe*. Jobs in French research organizations and universities are there if you look for them.

Looking requires understanding that almost all working scientists in France are civil servants, recruited in an annual series of national competitions, or *concours*. Most of them are on the payroll of the research organizations (22,000) and universities (37,000). Many also work for other public organizations such as the Commissariat à l'Energie Atomique (CEA) and Centre National d'Etudes Spatiales (CNES).

This universal job-for-life system also makes it almost impossible to create temporary posts for French scientists so post-doctoral fellowships within France are

Number of applications from each EC country for support from the EC Human Capital and Mobility Programme

Applications to from	Belgium	Denmark	Germany	Spain	France	Luxembourg	Greece	Ireland	Italy	Netherlands	Portugal	UK	EFTA	Total
Belgium			5	3	30				4	3		6		51
Denmark	1		2		5				2			7	1	18
Germany	10	6		8	85			4	10	7		53	5	188
Spain	11	6	16		58		2	1	15	10	1	59	3	182
France	15	2	14	10			3	2	9	9	3	34	6	107
Luxembourg	1				3							2		6
Greece	6	2	11	5	28				3	11		44	1	111
Ireland	2		2	2	9				1	3		21		40
Italy	10	4	13	6	45			2		11		23	12	126
Netherlands	2		4	3	18				1			6	1	35
Portugal	2		1		5					3		6	1	18
UK	1	3	6	11	23		2	1	6	4			5	62
EFTA	2	1	6		4				3			9		25

Data collected after July 1992 and January 1993 deadlines. The table does not include data concerning the success of the applications

rare. Nonetheless, opportunities do exist. In fact, the lack of mobility of French researchers combined with the inherent inertia of the *concours* means that active laboratories are often crying out for staff. They often take on foreign scientists on temporary contracts, one of the few legal options available.

Permanent positions. Scientists within the research organizations do not have to teach, and generally enjoy better research support than scientists at universities. With a FF 12.4 billion budget, the CNRS is Europe's largest fundamental research organization. It employs 11,417 researchers and 15,000 support staff. INSERM, the Institut National de la Santé et de la Recherche Médicale, is about a fifth the size of the CNRS. Its current budget (FF 2.3 billion) supports more than 2,000 researchers. INRA, the Institut National

de la Recherche Agronomique, this year has FF 3.1 billion from the research ministry; its 1,765 researchers make up around one fifth of its workforce.

The research organizations have allowed foreign scientists to compete for permanent positions since 1983. Almost 1,000 foreigners work in the CNRS (8.5%), over one-third of them in life sciences. The largest national group are British (96) followed by Americans (76) and Germans (68); over half the total are European nationals (three-quarters from EEC states). INSERM employs around 150 foreign scientists (6.7%), and INRA 70 (4%). Almost one-fifth (55) of permanent scientists at the cosmopolitan Institut Pasteur — which appoints its own staff — are also non French.

Foreign recruitment is growing: last year, one quarter of all new CNRS re-

cruits (around 500) were non-French. Most openings are at the junior grade of CR (there are five grades: CR1, CR2, DR1, DR2 and DRE, where CR means *chargé de recherche*, DR, *directeur de recherche*, and E, *exceptionnel*). Poor prospects for promotion from CR to DR have been a major source of discontent, but a wave of promotions — almost 4,000 between 1900 and 1992 — has improved the situation. Foreign postdocs also appear well qualified to enter at senior positions, last year they accounted for half those recruited at DR1.

ATIPÉ grants (Action Thématique et Incitative sur Programme et Equipes) are one of the most attractive routes into the CNRS. These provide successful applicants with extra funding for equipment and supplies for the first three years, to help them quickly establish new teams, working on priority themes. What's more, the researcher's entry to the CNRS (required by law) at the next *concours* becomes a formality.

ATIPES available this year include: compartmentation and functional organisation of eukaryotic cells (deadline 31 October); virology (except retrovirology) (1 November); developmental biology (15 October); and biomolecular engineering (part of the IMABIO programme, based at centres in Grenoble, Lyon and Strasbourg). The CNRS plans further ATIPES on "integrative molecular dynamics," ecology, and microbiology.

Foreign faculty. Not all university positions are open to foreign scientists. Permanent positions at the senior grade of *professeur* (15,204 in 1992) are reserved for French nationals. Foreign scientists may apply for the equivalent *professeur associé* grade, but this is a temporary position; foreigners held 342 such positions in 1992 (2.3%). Foreign scientists can apply for the junior grade of *maître de conférence*, and in 1992 held 1,210 (5.5%) of such posts (22,340); they also took 15% of all new positions. Recruitment is controlled by a national committee which allocates posts to each university, and a university committee which fills them. University committees may discriminate in favour of local candidates.

France's centralized universities are often synonymous with bursting classrooms, high teaching loads and poor research resources. An ongoing recruitment campaign is gradually reducing teaching hours, and the recent merger of the ministries of higher education and research might bring greater university autonomy and improve the allocation of research funding. The research resources of a university laboratory also depend on its status. Mixed university/research organization laboratories are generally the best equipped. University labs funded by the DRED committee (Direction de la Recherche et des Etudes Doctorales)

ORGANIZATIONS OFFERING POSTDOCTORAL FELLOWSHIPS TENABLE IN FRANCE

Political refugees

Entraide Universitaire Française, Comité Française de l'Entraide Universitaire Mondiale, 40 rue Rouelle, 75015 Paris, France

All nationalities

Ministère des Affaires Etrangères, "Séjours Scientifiques de Longue Durée" — DDCSTE, 34 rue de Prouse, 75775 Paris Cedex 16, France

Ministère de l'Enseignement Supérieur et de la Recherche, Délégation des affaires internationales, 1 rue Descartes, 75321 Paris Cedex 05, France

Agence Nationale de Recherches sur le SIDA, 66 bis avenue Jean-Moulin, 75014 Paris, France

Association Claude Bernard, 3 avenue Victoria, 75004 Paris, France

Association pour la Recherche sur le Cancer, 7 rue Guy Mocquet, BP3 98401, Villejuif Cedex, France

Fondation Fyssen, 194 rue de Rivoli, 75001 Paris, France

Fondation pour la recherche médicale, 54 rue de Varenne, 75007 Paris, France

Fondation pour la recherche thérapeutique, Commission scientifique, 25 rue Montevideo, 75116 Paris, France

Fondation Simone et Cino Del Duca, 10 rue Alfred de Vigny, 75008 Paris, France

Union Internationale Contre le Cancer, 3 rue de Conseil Général, 1205 Geneva, Switzerland

Member countries of the Agence de Coopération Culturelle et Technique (ACCT)

Agence de Coopération Culturelle et Technique, 13 Quai André Citroën, 75015 Paris, France

Member countries of the Association des Universités Partiellement ou Entièrement de Langue Française (AUPELF)

AUPELF, 192 bld Saint-Germain, 75007 Paris, France

Member countries of the United Nations Economic Commission for Africa (CEA)

Chef de l'administration publique, Division de la gestion et de la main-d'oeuvre, PO Box 3001, Addis Ababa, Ethiopia

Members of the European Molecular Biology Organisation (EMBO)

European Molecular Biology Organisation, Postfach 102240 6900 Heidelberg, Germany

Eastern and Central Europe

Ministère de l'Enseignement Supérieur et de la Recherche, Délégation des affaires internationales, 1 rue Descartes, 75321 Paris Cedex 05, France

European Science Foundation (ESF), 1 quai Lezay Marnesia, 67000 Strasbourg, France

Wellcome Trust, 1 Park Square, London NW1 4LJ, UK

Institut Candia, Centre de Recherche International, 10 rue J. J. Rousseau, 94200 Ivry/Seine, France

Commission of the European Communities, 200 rue de la Loi, B-1049 Brussels, Belgium

Member states of the Food and Agriculture Association (FAO)

Food and Agriculture Association, via delle terme di Caracalla, 00100 Rome, Italy

OECD member states

OECD, Direction de l'alimentation et de l'agriculture, 2 rue Pascal André, 75775 Paris Cedex 16, France

NATO member countries

Prof. Saint Paul, CNAM, 2 rue Conté, 75141 Paris Cedex 03, France

UNESCO member countries and associates

UNESCO, 9 place de Fontenoy, 75007 Paris, France

Canada/European Community/Japan/USA

International Human Frontier Science Programme Organisation, Tour Europe, 20 place des Halles, 67054 Strasbourg Cedex, France

Developing countries

Fondation Internationale pour la Science, Secrétariat, Sibyllégatan 47 S-114 42 Stockholm, Sweden

Spain

Ministerio de Educacion Y Ciencia Direccion General de Investigacion Cientifica, Calle Serrano 150, 28006 Madrid, Spain

United Kingdom

Royal Society (European Science Exchange Programme), 6 Carlton House Terrace, London SW1 5AG, UK

International Branch, AFRC, Polaris House, North Star Avenue, Swindon SN2 1VH, UK

Sweden

Institut Suedois, Svenska Onstitutet Service des bourses, Box 7439 10391 Stockholm, Sweden

Canada/Israel/United States

Ministère des Affaires Etrangères (Chateaubriand grants), Apply to science section of local French Embassy

within the ministry of education also have reasonable resources. Laboratories that do not fall into either category may have poor research means.

French laboratories have no mechanism to appoint temporary staff themselves (it's illegal). The research organizations side-step this problem by leaving some permanent positions unfilled, and letting these out as fellowships to foreign scientists. In 1991, the CNRS used such "*postes rouges*" to support 567 foreign "*professeurs associés*" for 3- to 12-month stays. In 1986, INSERM started to offer 6- to 11-month postdoctoral fellowships, renewable once (*postes verts*) and 6- to 12-month grants for sabbaticals by senior scientists (*postes oranges*). INSERM's emphasis is now on postdoctoral fellowships; between 1986 and 1991 it increased the number of these from 21 to 117, while reducing the number of *postes oranges* fellowships from 40 to 16. Associate professorships are sometimes advertised, but are often arranged directly with a laboratory; it may be worth contacting French labs with a view to submitting a joint application.

The CNRS also funds 95 European co-operation programmes (PICs), and several associated labs (LEAs, Laboratoires Européens Associés) such as one on astronomy between the Paris Astrophysical Institute, Cambridge University Institute of Astronomy (UK) and Leiden University Observatory (the Netherlands). Such programmes set money aside for collaboration and scientists thus avoid having to reapply for every visit.

Various French associations and medical charities sponsor small numbers of fellowships (see table); these are rarely advertised in international scientific journals. The French foreign ministry also offers up to 40 one-year (renewable once) postdoctoral fellowships — *séjours scientifiques de longue durée* — which provide a tax-free monthly salary of FF 12,000, and social security coverage. The ministry of higher education and research funds up to 60 1-to-6 month fellowships.

Many postdoctoral fellowships funded by outside organizations are tenable in France. The Royal Society last year funded 20 fellowships in France under its European Science Exchange Programme, while the European Commission funded 83 under its Human Capital and Mobility Programme. Fellowships are often available under agreements between France and an applicant's home country (the CNRS has 59 bilateral agreements with 39 countries); scientific attachés at French embassies can provide information on these.

In 1990, France began a series of fellowship programmes for eastern and central Europe. The ministry of higher education and research has accepted over 400 fundamental research scientists from these countries under a scheme which

allows for visits of 6 to 12 months (renewable once) and a FF 10,000 monthly salary; it also runs another scheme (BRITEST) which contributes 50% towards the costs of supporting researchers invited by a French company.

The CNRS has sponsored over 100 fellowships under eight twinning arrangements (*jumelages*) with laboratories in these countries. *Jumelages* allow small teams of researchers (5 to 15) to spend 6 months (renewable once, the following year) in French laboratories. Forty-four laboratory networks, each grouped around a specific theme, also allow for exchange of postgraduates and senior scientists. INSERM offers 40 1-to-3 month fellowships to researchers from eastern and central Europe, while INRA and the Commissariat à l'Énergie Atomique each funded visits by almost 100 scientists last year.

The research organizations say that they are convinced that they must recruit the best scientists, irrespective of nationality, if they are to be competitive. But one French organization has reservations. "More and more French companies and laboratories have joint ventures and collaborations with foreign partners, oblivious to the risks these bring," says an official at the Direction de la Surveillance du Territoire (DST), France's counter-espionage organization. Over the past two years, the DST has carried out a campaign among French companies and science organizations informing them of the risks of espionage and the precautions needed. The CEA vets and monitors visiting foreign scientists.

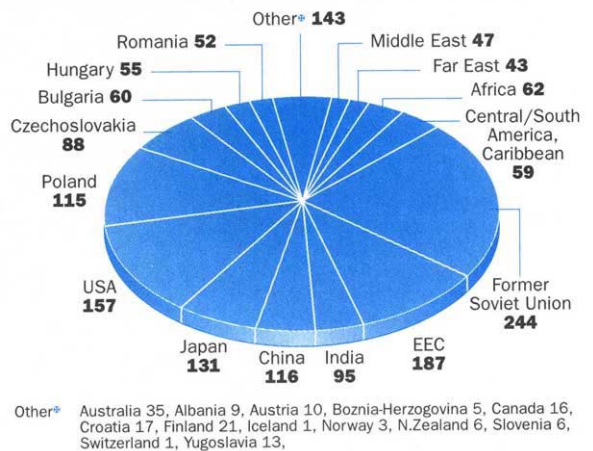
Germany

Germany is the richest country in Europe, with magnificent research facilities and generous research funds. But at the back of many minds lurks the question: is it wise to work as a foreigner in Germany with the recent upsurge of violent xenophobia?

The prospect that foreign scientists might be mistreated in their country has also been a great source of worry to the German research establishment. Last year all the major research organizations formed an alliance, representing virtually every scientist, to actively campaign against hostility towards foreigners. There was also a massive grass-roots reaction with millions of ordinary Germans lining the streets of every major city to express their outrage towards *Fremdenhass*, hatred of foreigners.

The truth is that there have been no reported physical attacks on foreign scientists in the safe haven of academia —

Humboldt Research Fellows in 1992 by country



although some have been subjected to verbal abuse on the streets. Nevertheless, the fear remains. This same discomfort is believed by many to underlie the relative lack of interest of Europeans in working in Germany under the Human Capital and Mobility Programme.

German science is truly international, and every German scientist, at least from the old *Länder*, is proficient in English. It cannot be said that every non-German scientist is proficient in German and, as in all European countries, the language barrier is often seen as a deterrent. It needn't be. A national programme offers foreigners cheap classes in German, and many employers offer other opportunities to help settle into the language and culture.

Where do scientists work? In direct contrast to the highly centralized French system, federalized Germany has devolved responsibility for universities and in part for research institutes to its 16 *Länder*. This means that central statistics are not always collected and information in many cases may only be available from individual research institutes.

There are five pillars to German research: the universities, the national research centres, the Max Planck institutes, the Fraunhofer institutes, and Blue List institutes which do not fall into any other category but nonetheless do research 'of national interest'.

For short-term fellowships to work in any of these laboratories, young scientists may turn to a number of different sources for support, including the EC, the Max Planck Society and many other private foundations (see table). The German science council, the DFG, has no special programme for funding foreign postdocs, but does provide short-term grants (1-3 months) for foreign scientists in certain countries to come to Germany, for example, to learn a new laboratory technique.

The most renowned source of support for foreign academics is the Alexander von Humboldt Foundation, established in 1860 to promote international scientific co-operation. Disbanded after the war, it

ORGANIZATIONS OFFERING POSTDOCTORAL FELLOWSHIPS TENABLE IN GERMANY

AIESEC	Association Internationale des Etudiants en Sciences Economiques et Commerciales	Gdu.KBSt	Gottlieb Daimier-und Karl Benz-Stiftung
AvH	Deutsches Komitee der AIESEC e.V.	HHS	Heinrich-Hertz-Stiftung
BIF	Alexander von Humboldt Foundation	HSS	Hanns-Seidel-Stiftung
BC	Boehringer Ingelheim Fonds	IAESTE	International Association for the Exchange of Students for Technical Experience
CDG	The British Council		Deutsches Komitee der IAESTE
CERN	Carl Duisberg Gesellschaft e.V.	KAAD	Katholischer Akademischer Ausländer-Dienst
CuW	Europäisches Laboratorium für Teilchenphysik	KEG	Kommission der Europäischen Gemeinschaften
DAAD	Cusanuswerk	KAS	Konrad-Adenauer-Stiftung e.V.
DFA	Deutscher Akademischer Austauschdienst e.V.	KminL	Kultusministerien der Länder in der Bundesrepublik
DFG	Deutscher Famulanten-austausch	LW	Deutsches Nationalkomitee des Lutherischen Weltbundes
DFHK	Deutsche Forschungsgemeinschaft	MSK	Minerva Stipendien-Komitee
DFJW	Deutsche-Französische Hochschule	MSS	Dr Mildred Scheel Stiftung für Krebsforschung
DREB	Hochschulkoileg	OBS	Otto Benecke Stiftung
DW	Deutsch-Französisches Jugendwerk	OSW	Ökumenisches Studienwerk e.V.
ES	Deutscher Rat der Europäischen Bewegung	PAD	Pädagogischer Austauschdienst
ESIV	Diakonisches Werk der EKD	SVR	Stiftungsverband Regenbogen
EUSIL	Eidgenössische Stipendienkommission für ausländische Studierende	StdV	Studienstiftung des Deutschen Volkes
FES	Evangelisches Studienwerk Villigst	SOG	Südosteuropa-Gesellschaft
FNS	Eurosilva	WUS	World University Service
FTS	Friedrich-Ebert-Stiftung	BAioG	Bundesausbildungsförderungsgesetz
FULKOM	Friedrich-Naumann-Stiftung	GFG	Graduiertenförderung
	Fritz Thyssen Stiftung		For further information, contact the DAAD.
	Fulbright-Kommission		

was re-established in 1953 to forge international links and co-operation in science, something very important to the devastated German economy. It is mostly government funded. It offers long-term fellowships of between 6 months and 2 years and supports around 10 per cent of all foreign postdocs in Germany. The Humboldt Foundation does not see itself as a match-making bureau. Candidates must find their own contacts in universities or research institutes, and arrange their own projects before applying for a grant.

Those lucky enough to receive a Humboldt fellowship (last year 3,000 applied for only 500 places) find themselves very well looked after. Although they must be proficient in English, the Humboldt Foundation finances intensive German courses for postdocs and their spouses for 2 to 4 months before the start of the fellowship. It also finances equipment and books, where necessary, to help set up a laboratory in the home country, and a return to Germany for a short spell. Around 15 per cent of the budget is allocated to this 'aftercare', which mainly supports poor countries. Last year 70 per cent of all scientific equipment, 60 per cent of all books, and 50 per cent of all re-invitations, went to eastern Europe.

The geographical breakdown of Humboldt fellowships (see below) has changed considerably during the last four years with the opening up of the communist bloc. The Humboldt Foundation was given more money in the late 1980s to help sponsor the increasing number of applications for scholarships from eastern and central Europe. Applications from Rus-

sia, for example, increased from 50 in 1988 to 400 in 1992. But recession soon forced the total numbers back to 500.

The Humboldt Foundation has a sister organization, the Deutscher Akademischer Austauschdienst (DAAD), whose DM146 million programme supports 5,500 short-term scholarships (three months to one year) for students and graduates, for either study or research. Most contacts are made through DAAD's extensive network system, but individuals may apply via their German embassies.

There are also plenty of permanent opportunities for foreign scientists in German universities and research institutes, "German faculty is extremely open to foreigners", says Gerhard Maass of the Humboldt Foundation. "Over the years we have sponsored 5,000 people to come to Germany, and 120 of them now have full professorships." More than five per cent (over 5,000) of university academic staff are non-nationals. The great majority come from other European countries

Useful addresses:

Arbeitsgemeinschaft Forschungseinrichtungen Blaue, Liste (AG-BL), Ardeystrasse 67, D-4600 Dortmund 1. Phone (0231) 1084-204/fax (0231) 1084-326
Arbeitsgemeinschaft der Grossforschungseinrichtungen (AGF), national research centres, Ahnstrasse 45 (Wissenschaftszentrum), D-53175 Bonn. Phone (0228) 37674-1/fax (0228) 37674-4
Deutscher Akademischer Austauschdienst (DAAD), Kennedyallee 50, D-5300 Bonn 2. Phone (0228) 882-0/fax (0228) 882-365
Fraunhofer Society, Leonrodstrasse 54, D-8000 Munich 19. Phone (089) 1205-0/fax (089) 1205-317
Alexander von Humboldt Foundation, Jean-Paul-Strasse 12, D-5300 Bonn 2. Phone (0228) 833-0/fax (0228) 833-199
Max Planck Society, Postfach 101062, D-8000 Munich 2. Phone (089) 21081/fax (089) 229850

but a surprisingly high proportion (27 per cent) come from Asia. Less than eight per cent come from north America (see piechart). Many, but not all, academic jobs are advertised internationally. The most reliable place to monitor university posts is the universities' magazine, *Das Hochschulmagazin* (DUZ).

The Max Planck Society employs 11,000 staff of which 790 are foreign scientists. Like all Max Planck scientists, most are employed on long-term contracts of around five years and are entitled to work for a total of around eight years. But 29 are research directors — a high proportion of the total number (190) of tenured directorships. The Max Planck Society also has 4,270 short-term guest scientists who work for around 6 months at one of the 97 institutes and research groups. Of these, 1,725 are foreigners. Most come from Europe (23 per cent from EC countries; 35 per cent from the rest of Europe), but many come from North America (14 per cent). Max Planck institutes advertise their post openings internationally, but 'head-hunt' institute directors.

There are 16 national research centres, which conduct research which cannot be carried out in universities because it requires too many personnel or too expensive equipment. Between them they have over 23,000 employees, at least half of whom are scientists or engineers. Each centre is independent and central statistics of foreigners are not collected. But individual estimates suggest that the proportion of foreign staff members varies considerably between the sixteen. The very special skills required of the 1,000 scientists at the nuclear research centre in Karlsruhe (KfK) means that nearly a third are foreigners. By contrast only 80 of the 1,620 scientists at the air and space research centre (DLR) in Cologne are foreigners. In addition to the permanent staff, a stream of foreign guest scientists pass through the centres for short periods.

The Fraunhofer Society has 7,400 staff, of whom 2,830 are scientists. Four per cent (105) of the scientists are foreigners, two thirds of whom are European. Many are recruited by personal contact, but positions are also advertised internationally.

Blue List institutes employ 9,000 in their 82 establishments, 34 of which are in the new *Länder*. A complete list of the institutes is available from the Blue List Society (AG-BL). Again, opportunities — for temporary or permanent positions — vary widely according to institute.

The pharmaceutical and electronics industries also play host to foreign researchers. The extensive research facilities of Frankfurt-based Hoechst and Munich-based Siemens are 4-5 per cent staffed by foreigners. The German international giants Boehringer-Mannheim, Bayer and BASF have similar open-door policies. □